ENHANCED BIOHYDROGEN PRODUCTION AND SUBSTRATE UTILIZATION BY CO-CULTURE FERMENTATION WITH REDUCED EXTRACELLULAR ELECTRON SHUTTLES

BY

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DISSERTATION

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ABSTRACT

Hydrogen is a promising energy carrier due to no greenhouse gas (GHG) emission during combustion and the highest conversion efficiency in fuel cells and highest energy content per unit mass compared to carbon-based energy carriers. However, three key challenges for large-scale biohydrogen production are to increase i) the hydrogen production rate, ii) the hydrogen molar yield, and iii) the extent of substrate utilization. A co-culture system of *C. beijerinckii* and *G. metallireducens* with extracellular electron shuttles was developed and evaluated for improved biohydrogen production.

To enhance biohydrogen production, *Clostridium beijerinckii* was co-cultured with *Geobacter metallireducens* in the presence of the reduced extracellular electron shuttle anthrahydroquinone-2, 6-disulfonate (AH_2QDS). In the co-culture fermentation system, increases of up to 52.3% for maximum cumulative hydrogen production, 38.4% for specific hydrogen production rate, 15.4% for substrate utilization rate, and 39.0% for substrate utilization extent were observed compared to a pure culture of *C. beijerinckii* without AH_2QDS. *G. metallireducens* grew in the co-culture system, resulting in a decrease in acetate concentration under co-culture conditions and a presumed regeneration of AH_2QDS from AQDS. These co-culture results demonstrate metabolic crosstalk between the fermentative bacterium *C. beijerinckii* and the respiratory bacterium *G. metallireducens* and suggest a strategy for industrial biohydrogen production.

This co-culture system was further applied to ferment complex substrates from hydrolysates of lignocellulosic biomass as well as to utilize compounds including indigo dye, juglone, lawsone, fulvic acids and humic acids as alternative extracellular electron shuttles. The observed improvements in utilization of lignocellulosic hydrolysates and particularly utilization of xylose

support the feasibility of applying this co-culture system to lignocellulosic hydrolysates, especially xylose-rich ones, in industry. In addition, the replacement of AH_2QDS by alternative extracellular electron shuttles, such as humic acids, makes the co-culture with extracellular electron shuttle system more economical and flexible.

Keywords: biohydrogen, extracellular electron shuttles, co-culture, syntrophy, lignocellulosic hydrolysates, fermentation

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CHAPTER 1 INTRODUCTION

Biofuels attract more and more attention as a renewable source of energy. Current energy is mostly provided by fossil fuels such as coal, petroleum, and natural gas that are non-renewable. Energy Information Administration (EIA) reported that world energy demand is growing about 0.6% in the Organization for Economic Cooperation and Development (OECD) member economies and 2.3% per year in the non-OECD economies from 2008 to 2035 (DOE/EIA-0484 (2011)). The estimated production years left for the proven reserves of oil, coal and natural gas are 35, 107, and 37 years, respectively. The increasing energy demand promotes the development of renewable energy including biofuels. Biofuels are also considered to be environmental friendly due to less emission of greenhouse gas (*e.g.* carbon dioxide) and air pollutants (*e.g.* sulfur dioxide) (Verma et al. 2011). In addition, some emerging biofuel technologies can consume biological wastes as feedstocks or integrate with wastewater treatment processes, addressing environmental protection and energy crisis issues simultaneously. Worldwide biofuel production grew 14% to 59 million tonnes of oil equivalent (1.9 million barrels daily on a volumetric basis) in 2010 (BP statistical review, 2011). According to the International Energy Agency, biofuels have the potential to meet more than one-fourth of global demand for transportation fuels by 2050 (Swann 2008).

The development of different approaches for biofuel production is described in four generations. The 1st generation biofuels utilized food crops such as sugar cane, starch, vegetable oil, and animal fat as the feedstocks (Walker 2011). This is a mature industry and the feedstocks contain high sugar/fatty acids content, which makes the substrate utilization more efficient. However, energy production is in direct competition with food production (Cheng and Timilsina 2011). The 2nd generation uses lignocelluloses such as forest products, agricultural residues, and

energy crops as feedstocks (Walker 2011). It expands the feedstock resources for biofuel production, but is currently limited by the yield and overall efficiency of fuel production (Weber et al. 2010). The 3rd generation suggests genetically modified crops (Taylor 2008) or algae (Parman et al. 2011) as substrates with a higher yield than lignocelluloses. However, the environmental impact concerns of genetically modified crops (Conner et al. 2003) and the high cost due to the growth and harvest efficiency for algae (Norsker et al. 2011) require more studies. The 4th generation promotes the idea of combining the biofuel process (1st, 2nd or 3rd generation) with carbon storage and sequestration to achieve a negative carbon footprint, which makes the overall process more cost-effective and efficient. Environment-enhancing bioenergy production can address both environment and energy security issues. For example, biomass-to-bioenergy (biohydrogen, bioethanol and bio-oil) process has the potential to utilize waste as feedstocks. The integration of algae cultivation with municipal wastewater treatment process or industrial CO_2 emissions from coal-fired power plants can solve environment protection and biofuel production simultaneously (McGinn et al. 2011). Some researchers use this strategy to save up to 50% of operational cost for wastewater treatment and produce abundant algae for extra biofuel production (personal communication with Dr. Lance Schideman's group in University of Illinois at Urbana-Champaign,). Current research for biofuel production focuses on increasing yield and substrate utilization from 2nd generation feedstocks (lignocellulose), decreasing costs of 3rd generation process and developing applicable combination strategies for 4th generation.

The form of biofuels includes solid (combustion of biomass), liquid (bio-alcohol, biodiesel, and bio-oil) and biogas (methane and biohydrogen). United States and Brazil are the world's top producers for bioethanol while Europe is the largest biodiesel producer (Kumar 2011). More and more research has focused on biobutanol (Ezeji et al. 2004) due to its higher energy content,

lower vapor pressure, easier storage under humid conditions, less corrosion and more compatibility to the existing infrastructures compared to bioethanol (Pfromm et al. 2010; Szulczyk 2010).

Hydrogen is also considered to be a promising energy carrier and maybe the ultimate biofuel in the long term because of no greenhouse gas (GHG) emission when combustion and the highest conversion efficiency in fuel cells and highest energy content per unit mass compared to carbon-based energy carriers (Bartacek et al. 2007; Kapdan and Kargi 2006; Nath and Das 2004; Sinha and Pandey 2011; Zhang 2011). Compared to physical/chemical approaches for hydrogen production, hydrogen produced from biomass, or biohydrogen, is more environmentally sustainable because of less emission of greenhouse gases and air pollutants, less energy intensive during production (Kapdan and Kargi 2006; Oh et al. 2004; Saxena et al. 2009; Ust'ak et al. 2007) and less operational complications at ambient temperature and atmospheric pressure (Das and Veziroglu 2008). Biohydrogen from dark fermentation shows advantages in reactor design and operation as well as production rates over other biohydrogen production methods (Turner et al. 2008).

Biohydrogen, bioethanol and biobutanol production as 2nd generation biofuels are based on lignocellulosic biomass where biological fuel production is typically preceded by physical/chemical/thermal pretreatment (de Vrije et al. 2002). Pentoses (*e.g.* xylose) and hexoses (*e.g.* glucose) are the major lignocellulosic hydrolysates (Ahring et al. 1996; Sun and Cheng 2002). Compared to glucose fermentation, the relatively low hydrogen molar yield, hydrogen production rate and the substrate utilization from xylose limit the application of biohydrogen production. Currently, the key challenges for large scale application of biohydrogen are how to increase the hydrogen production rate and molar yield as well as the extent of substrate

utilization (Kim et al. 2010; Kuhad et al. 2011; Sarkar et al. 2012).

Clostridium is the major genus for biohydrogen-producing from dark fermentation, which converts carbohydrates to metabolic byproducts without light in anaerobic conditions. *Clostridium beijerinckii* is a robust biohydrogen-generating fermenter, based on its specific hydrogen production rate and yield (Benemann 1996; Jeong et al. 2008). Previous study found extracellular electron shuttles (EES) such as reduced anthraquinone-1, 6-disulfonate (AH_2QDS) enhanced hydrogen production in *C. beijerinckii* fermentation by affecting the intracellular $NADH/NAD^+$ ratio and shifting metabolic pathways (Hatch and Finneran 2008; Ye et al. 2011). Electron shuttling compounds cycle between oxidized and reduced forms and intracellular electron shuttles such as ubiquinone, ferredoxin and cytochromes play an essential role in electron transport chain of microorganisms (White 2000). Several research applied EES to biofuel production (Hatch and Finneran 2008; Ye et al. 2011), bioremediation (Kwon and Finneran 2008; Lovley et al. 1998; Stams et al. 2006), wastewater treatment (Watanabe et al. 2009), microbial fuel cells (Aranda-Tamaura et al. 2007), and fermentation (Girbal et al. 1995a; Girbal et al. 1995b; Peguin et al. 1994; Peguin and Soucaille 1995; Zhang et al. 2009).

However, the reduced form AH_2QDS is unstable under aerobic conditions and oxidized to AQDS during the fermentation. In addition, chemical regeneration of AH_2QDS consumes energy. For application in a continuous reactor, efficient processes are needed to regenerate AH_2QDS. We suggest overcoming this obstacle through co-culture with *Geobacter metallireducens*. Respiratory *G. metallireducens* is able to oxidize the fermentation products acetate and butyrate using AQDS (oxidized form) as an electron acceptor to regenerate AH_2QDS biologically (Aklujkar et al. 2009; Kwon and Finneran 2006; Kwon and Finneran 2008; Lovley et al. 1993; Wolf et al. 2009b).

The overall objective for this research is to improve fermentative biohydrogen production (hydrogen molar yield, production rate and substrate utilization) from complex substrates. Syntrophy in a co-culture of *C. beijerinckii* and *G. metallireducens* with EES is proposed and evaluated (Figure 1.1). C. *beijerinckii* ferments xylose to produce H_2 and acetate/butyrate. *G. metallireducens* oxidizes acetate/butyrate to generate AH_2QDS, which can be taken up by *C. beijerinckii* to promote H_2 production either as an electron donor (4) or by modifying internal metabolism (*e.g.*, $NADH/NAD^+$, (5)). Therefore, in the presence of reduced extracellular electron shuttles, syntrophy of the two cultures is expected, increasing fermentative biohydrogen production. To evaluate the feasibility of co-culture in the presence of EES system as a cost-effective and practical strategy for biohydrogen production, the co-culture with EES was then applied to the fermentation of complex substrates such as hydrolysates from the thermochemical pretreatment of lignocellulosic biomass (*Miscanthus*) by diluted acids, which contains different ratios of glucose and xylose as well as fermentation inhibitors. The possibility to utilize economical EES was also investigated. EES such as juglone (Wolf et al. 2009b), lawsone (Lovley et al. 1998; Wolf et al. 2009b), fulvic acids (Fulton et al. 2004; Wolf et al. 2009b) and humic acids (Wolf et al. 2009b) have been used as electron transfer mediators for bioremediation by *G. metallireducens*. They are expected to play the same role as AH_2QDS in co-culture system.

Chapter 2 reviews the literature on current progress and difficulties for biohydrogen production. Chapter 3 develops and evaluates the syntrophy in the proposed co-culture system with extracellular electron shuttle (EES) for the enhanced biohydrogen production using a simple substrate and model EES. Chapter 4 applies the co-culture system using complex substrate for fermentation and different extracellular electron shuttles. The general conclusions and future

work are presented in Chapter 5.

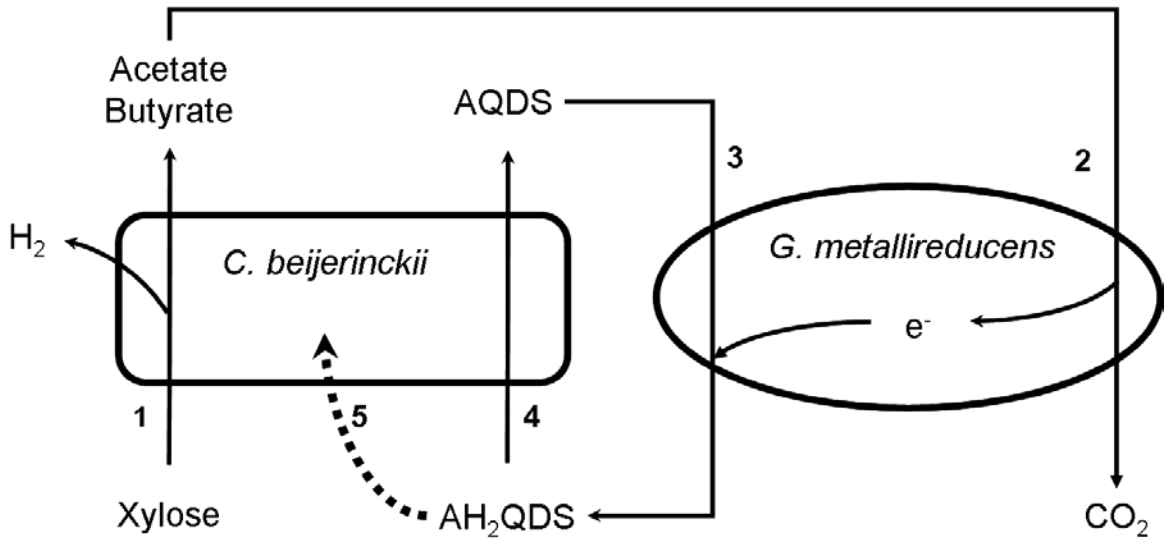

Figure 1.1 Conceptual model for the co-culture fermentation. 1) xylose is fermented to acetate and butyrate by *C. beijerinckii*, 2) acetate or butyrate are oxidized by *G. metallireducens*, (3) electrons from (2) are used by *G. metallireducens* to reduce AQDS to AH_2QDS, 4) *C. beijerinckii* oxidizes AH_2QDS during the fermentation process, and 5) AH_2QDS further increases hydrogen production through effects on *C. beijerinckii* metabolism.

CHAPTER 2 LITERATURE REVIEW

This chapter reviews the background knowledge, major parameters and key challenges for the biohydrogen, especially the fermentative biohydrogen production. The research on the electron shuttles and syntrophy are also summarized.

2.1 Biohydrogen production approaches

Biohydrogen production can be classified into three categories: biophotolysis of water, photo fermentation and dark fermentation from organic compounds (Bartacek et al. 2007; Benemann 1996; Das and Veziroglu 2001; Das and Veziroglu 2008; Hallenbeck and Benemann 2002; Levin et al. 2004b; Saxena et al. 2009). Microbes involved in these approaches are algae, cyanobacteria, photosynthetic bacteria and fermentative bacteria (Nandi and Sengupta 1998; Yang et al. 2006).

2.1.1 Biophotolysis

Biophotolysis converts solar energy into chemical energy by splitting water under sunlight. It includes two sub-categories: direct biophotolysis by green algae (Burgess et al. 2011; Melis and Happe 2001; Ust'ak et al. 2007) and indirect biophotolysis by blue-green algae (cyanobacteria) (Lopes Pinto et al. 2002; Stevens et al. 1973).

The reaction for direct biophotolysis is:

$$2H_2O \xrightarrow{\text{light energy}} 2H_2 + O_2 \quad (2.1)$$

Indirect biophotolysis involves two steps:

$$12H_2O + 6CO_2 \xrightarrow{\text{light energy}} C_6H_{12}O_6 + 6O_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 12H_2O \xrightarrow{\text{light energy}} 12H_2 + 6CO_2 \quad (2.3)$$

2.1.2 Photo fermentation

In photo fermentation (also known as photodecomposition), purple non-sulfur bacteria (Chen et al. 2008a; Zhu et al. 2007) produce H_2 from light energy and reduced compounds (organic acids) with the presence of nitrogenase under nitrogen-deficient conditions. The overall biochemical pathways can be described as follows (Ye 2011):

$$(CH_2O)_2 + 2H_2O \xrightarrow{\text{light energy}} 4H_2 + 2CO_2 \quad (2.4)$$

Certain photoheterotrophic (photosynthetic) bacteria within the superfamily *Rhodospirillaceae* can also grow without light to produce H_2 and generate ATP simultaneously via the following microbial water-gas shift reaction (Oh et al. 2003; Uffen 1983), which is a thermodynamically favorable process with a negative ΔG^0 value:

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (2.5)$$

$$\Delta G^0 = -20.029 kJ / mol$$

2.1.3 Dark fermentation

Hydrogen can also be produced without light by anaerobic bacteria grown on carbohydrate-rich substrates with volatile fatty acids (VFA) as co-products (Benemann 1996). Typical reactions using glucose as substrates include:

$$C_6H_{12}O_6 \longrightarrow C_4H_8O_2 + 2H_2 + 2CO_2 \quad (2.6)$$

$$\Delta G^0 = -224.128 kJ / mol$$

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2C_2H_4O_2 + 4H_2 + 2CO_2 \quad (2.7)$$

$$\Delta G^0 = -135.962 kJ / mol$$

Microorganisms involved in the dark fermentation include species of *Enterobacter* (Long et

al. 2010; Tanisho et al. 1989; Zhang et al. 2009), *Bacillus* (Chang et al. 2008; Kalia et al. 1994) and *Clostridium* (Brosseau and Zajic 1982; Chen et al. 2008b; Collet et al. 2004; Fritsch et al. 2008; Skonieczny and Yargeau 2009; Zhang et al. 2012a). The growth of fermentative bacteria needs two key factors: energy generation and the balance of redox reactions (White 2000). Large amounts of reduced electron equivalents (NADH) are generated during the glycolysis or pentose-phosphate pathway (Temudo et al. 2009), which need to be reoxidized. In anaerobic condition, protons, which can be reduced to H_2, can act as the electron acceptor for NADH oxidation. There are two major enzyme systems for hydrogen production (Hallenbeck 2005):

1. Pyruvate ferredoxin oxidoreducatase (PFOR):

$$\text{Pyruvate} + \text{CoA} + \text{Fd}\ (\text{ox}) \rightarrow \text{Acetyl} - \text{CoA} + \text{Fd}\ (\text{red}) \tag{2.8}$$

$$\text{Hydrogenase}: \text{Fd}\ (\text{red}) + \text{H}^+ \rightarrow \text{H}_2 + \text{Fd}\ (\text{ox}) \tag{2.9}$$

2. Pyruvate formate lyase (PFL):

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{Acetyl} - \text{CoA} + \text{formate} \tag{2.10}$$

$$\text{Hydrogenase}: \text{formate} \rightarrow \text{H}_2 + \text{CO}_2 \tag{2.11}$$

Clostridium produces hydrogen via the first enzyme system, while the latter is common in enteric bacteria such as *Enterobacter* (Hallenbeck 2005; Levin et al. 2004a).

Table 2.1 summarizes the advantages and disadvantages of different biohydrogen production processes. To date, dark fermentation process has advantages of process simplicity, utilization of waste, lower energy requirements and higher hydrogen production rate compared to other biohydrogen approaches. Genetically modified pure culture (Jones 2008; Li et al. 2010; Maeda et al. 2008), co-cultures (Fang et al. 2006; Yokoi et al. 1998)) and mixed cultures from anaerobic digesters (Cheong and Hansen 2006; Fakhru'l-Razi et al. 2005; Fang and Liu 2002; Herbert H. P. Fang 2002; Jun et al. 2008; Khanal et al. 2006; Kongjan et al. 2009; Lin et al. 2008; Monmoto et

al. 2004; Mu et al. 2006a; Yokoi et al. 2002; Zhang et al. 2006b; Zuo et al. 2005) have been studied for the dark fermentative biohydrogen production.

2.2 Dark fermentative biohydrogen production

2.2.1 *Clostridium* fermentation pathway

Clostridium sp. are gram-positive, rod-shaped, spore-forming, strict anaerobes (Collins et al. 1994; Keis et al. 1995; Keis et al. 2001) and are frequently used for biohydrogen production in the literature (Hsiao et al. 2009; Mitchell et al. 2009; Oh et al. 2009; Ye 2011; Zhao et al. 2011). Figure 2.1 shows the fermentative pathway in *Clostridium sp* using glucose and xylose as model substrate. Hydrogen is produced from reduced ferredoxin via pyruvate ferredoxin oxidoreducatase system.

Glucose or xylose is first converted to pyruvate through glycolysis or pentose phosphate pathway, respectively, with production of the reduced form of nicotinamide adenine dinucleotide (NADH). Pyruvate is then converted to acetyl-CoA, CO_2 and H_2 by pyruvate: ferredoxin oxidoreductase and hydrogenase. Acetyl-CoA can be further converted to intermediates such as acetate, butyrate, as well as acetone, butanol, and ethanol (known as ABE in industry). A typical *Clostridium* fermentation includes two phases: hydrogen and organic acids are produced during the exponential growth phase (acidogenic phase), while rapid alcohol production occurs in stationary growth phase (solventogenic phase).

2.2.2 Key challenges for fermentative biohydrogen production

There are mainly three key challenges for industrial application of biohydrogen production: hydrogen molar yield, hydrogen production rate and substrate utilization extent.

2.2.2.1 Hydrogen molar yield

Theoretically, the hydrogen molar yield is 12 mol H_2/mol glucose if the substrate is completely converted to CO_2 and H_2 (2.12).

$$C_6H_{12}O_6 + 12H_2O \longrightarrow 12H_2 + 6CO_2 \quad (2.12)$$

$$\Delta G^0 = 1397.202 kJ / mol$$

However, the fermentors need to generate enough ATP for cell growth and to reoxidize NADH produced during substrate level phosphorylation (SLP). Pyruvate, which is a key high-energy metabolite for ATP generation in cells, can be used to produce NAD^+. Pathways to produce hydrogen, butyrate, ethanol and butanol also can regenerate NAD^+ and save more pyruvate for energy generation through the PTA/ACK pathway (White 2000) with acetate as product (Figure 2.1). Therefore, the production of H_2, organic acids or solvents is necessary during fermentation, and the end products which can serve for NAD^+ regeneration are called electron sinks (White 2000). According to the literature, the theoretical biohydrogen molar yield for dark fermentation is between 2 mol H_2/mol glucose with butyrate as the sole byproduct (2.6) and 4 mol H_2/mol glucose with production of acetate as the associated organic acid (2.7). Experimental values reported so far are only around 2 mol H_2/mol glucose (Angenent et al. 2004; Lee and Rittmann 2009; Li and Fang 2007). The theoretical maximum hydrogen molar yield from xylose fermentation is 3.3 H_2/mol xylose with acetate as sole byproduct (2.13), but most reported values are much less than 2 mol H_2/mol xylose (Lin et al. 2008). Some reported dark fermentative hydrogen yields fermenting xylose, glucose or real substrates with either pure culture or mixed cultures are summarized in Table 2.2 (Nath and Das 2004; Sinha and Pandey 2011; Wang and Wan 2009).

$$C_5H_{10}O_5 + \frac{5}{3}H_2O \longrightarrow \frac{5}{3}C_2H_4O_2 + \frac{10}{3}H_2 + \frac{5}{3}CO_2 \quad (2.13)$$

$\Delta G^0 = -195.5 kJ / mol$

Fermentative H_2 production is coupled with the redox reaction of ferredoxin, which can exchange electrons with NADH/NAD^+ by the catalysis of NADH: ferredoxin oxidoreductase. Therefore, electron exchanges between NADH/NAD^+ and Fd (red)/(ox) pools are considered to be important for hydrogen production, and the intracellular NADH/NAD^+ ratio has been reported as an index for fermentative biohydrogen production.

2.2.2.2 Hydrogen production rate

Hydrogen production rate is important for commercial-scale application due to economic and practical consideration, since it is an important factor for bioreactor design. The bioreactor size will become impractically large for industrial application when the production rate is too low. Although the reported values vary widely (5.2-9310 ml H_2/l/h) and are in different units such as ml H_2/L/h and mmol H_2/L/h (Das and Veziroglu 2008; Levin et al. 2004b; Wang and Wan 2009; Ye et al. 2011), dark fermentation generally achieves higher hydrogen production rates than light-driven biohydrogen system. Thermophilic microorganisms generate hydrogen faster than mesophilic bacteria. However, the heat demands reduce the overall energy efficiency. The hydrogen production rates are affected by the microbes and operational conditions such as pH, temperature, and substrate (types and loading rates), as well as end-product accumulation.

2.2.2.3 Substrate utilization

Lignocellulosic biomass is considered to be a promising feedstock for biofuel production due to its abundance (*e.g.*, from agricultural residues, forestry wastes, municipal solid waste, paper pulp,

and fast-growing prairie grasses), low greenhouse gas emissions, and possible high energy output (Lee 1997; Petrus et al. 2006). After delignification (physical/chemical pretreatment to liberate cellulose and hemicellulose from the lignin) and hydrolysis (converting cellulose and hemicellulose to monosaccharides), glucose and xylose are the major products for further fermentation. Xylose is the second most abundant sugar in nature and accounts for approximately 30% of the total fermentable sugar from lignocellulose (Cu et al. 2009; Kumar et al. 2009). Compared to glucose, the substrate utilization of xylose is less efficient, probably because the pentose-phosphate pathway is less energy efficient than glycolysis and limited microbes can utilize xylose. Most reported xylose utilization efficiencies are less than 50% when fermented to hydrogen or ethanol/butanol (Kongjan et al. 2009; Prakasham et al. 2009). Pretreatment will also produce byproducts such as acetate, furfural and hydroxymethyl furfural (HMF), which can fermentation (Cao et al. 2010; Palmqvist and Hahn-Hägerdal 2000; Quéméneur et al. 2012; Tang et al. 2012; Van Ginkel and Logan 2005; Wang et al. 2008b; Zhang et al. 2012a).

2.2.3 Parameters affecting fermentative biohydrogen production

Fermentative biohydrogen production is influenced by many factors, including inocula, substrate, C/N ratio, pH, temperature, reactor operation modes, product inhibition, nitrogen, phosphate, and metal ions.

2.2.3.1 Strain development (Inocula)

Both pure culture and mixed cultures have been applied for fermentative hydrogen production. *Clostridium* and *Enterobacter* are the most widely studied options. Generally, *Clostridium* species can form spores for unfavorable environment and have a higher H_2 yield than

Enterobacter sp (Hawkes et al. 2002), especially when using xylose as a substrate (Levin et al. 2004b). The major advantage of *Enterobacter* sp. is their tolerance to oxygen (Bartacek et al. 2007). Mixed cultures from anaerobic sludge, with *Clostridium sp.* as dominant microbes, obtain attention currently because no sterile operation is needed, and microbial diversity can provide more metabolic pathways, which allows a larger range of substrates and adaptation to influent variations. However, pure culture fermentation is still important since it usually achieves higher hydrogen molar yield and it provides insight for fundamental metabolic pathway study. Genetically modified microorganisms (Jones 2008; Li et al. 2010; Maeda et al. 2008) and co-culture (Chang et al. 2008; Fang et al. 2006; Hsiao et al. 2009; Liu et al. 2008; Maintinguer et al. 2011; Wang et al. 2008a) have also been suggested as inocula to improve biohydrogen production.

2.2.3.2 Process Optimization

Process performance can be improved by adjusting the following parameters.

2.2.3.2.1 Reactor operation modes

Most studies are conducted in batch mode because of its simple operation and control. Different reactor configurations have been investigated, including chemostat (Crabbendam et al. 1985; Gapes et al. 1996; Guedon et al. 1999; Ueno et al. 1996), immobilized-cell reactor (Wu et al. 2008a; Wu et al. 2008b), fluidized bed reactor (FBR) (Koskinen et al. 2007), and membrane reactor (Oh et al. 2004). Theoretically, chemostats achieve higher productivity in prolonged periods than batch culture for growth associated products (McNeil and Harvey 2008; Stanbury et al. 2003). The major concerns for continuous system are how to maintain high concentration of culture and avoid shifting from the acidogenic pathway to the solventogenic pathway.

Immobilization can help to maintain high biomass concentration (Kim et al. 2005). Operational parameters such as hydraulic retention time (Fan et al. 2006; Hafez et al. 2009), solids retention time (Kim et al. 2008b), organic loading rate (OLR) (Kraemer and Bagley 2007; Shen et al. 2009), pH, and temperature are important to improve hydrogen production.

2.2.3.2.2 Substrate

Carbohydrates (*e.g.,* glucose, sucrose and starch) are widely used substrates for fermentative hydrogen production. Hexoses such as glucose and sucrose are favorable substrates for fermentation. A hydrogen molar yield of 2.7 mol H_2/mol glucose was reported in batch fermentation, and 6.12 mol H_2/mol sucrose in a continuous system (Wang and Wan 2009). Fermentation of pentoses (*e.g.* xylose) is less efficient and the microbes can utilize pentose are limited (Li et al. 2010; Lo et al. 2008; Long et al. 2010; Maintinguer et al. 2011). Lignocellulosic biomass is even more difficult to ferment, so that chemical/physical pretreatments to break down the crystal structure and release cellulose and hemicellulose from the lignin complex are necessary. In a certain range, hydrogen molar yield and production rate increase with substrate concentration. However, at much higher levels, the hydrogen production will slow down or further decrease due to the limitation of substrate utilization as well as product inhibition (Wang and Wan 2009).

2.2.3.2.3 pH

pH is considered to be a key parameter for fermentative hydrogen production. It influences the hydrogen molar yield and production rate significantly, probably due to the effects on the redox reaction involved and the enzymatic activity. However, there are conflicting reports about the optimal pH and how it affects the hydrogen production. Hydrogen production increases with

increasing pH, then decreases based on the review of Wang and Wan (2009). In mixed culture, lower pH is usually preferred, since pH< 5 inhibits the growth of H_2-consuming bacteria such as methanogens and acetogens (Fang and Liu 2002; Kim et al. 2004; Lee et al. 2002). However, in general, low pH is not favorable for cell growth and ATP generation for *Clostridium* pure culture (White 2000), and a pH < 5 can trigger the shift from the acidogenic pathway to the solventogenic pathway, decreasing hydrogen production. pH close to 7 was also observed for maximum hydrogenase activity (Baek et al. 2006). Amendment of sodium acetate was reported to improve solvent production by *C. beijerinckii* BA 101 (Chen and Blaschek 1999). For a certain pH value, uncontrolled pH (low buffer system) and controlled pH (acid/base addition or high buffer system) can lead to different results. Initial pH was also reported to affect the hydrogen production (Fangkum and Reungsang 2011; Kapdan and Kargi 2006).

2.2.3.2.4 Temperature

Temperature is another important factor for fermentative hydrogen production. In mixed culture, heat shock pretreatment (Kraemer and Bagley 2007; Lay et al. 2003) is widely accepted as a method to improve hydrogen production. Increased temperature will lead to a higher hydrogen production yield because of the inhibition of most methanogens as well as thermodynamic benefits for the reactions at thermophilic temperatures (Hallenbeck 2005). The optimal temperature for *Clostridium beijerinckii* pure culture is 37°C.

2.2.3.2.5 Product inhibition

High hydrogen partial pressure inhibits hydrogen production, according to thermodynamics and enzyme activity (Hawkes et al. 2002). High concentrations of CO_2 are also known to be inhibitory (Kraemer and Bagley 2006; Tanisho et al. 1998). Gas sparging with N_2 is widely

applied in large-scale reactors (Kim et al. 2006; Kraemer and Bagley 2006; Kraemer and Bagley 2007; Mizuno et al. 2000; Valdez-Vazquez et al. 2006). Logan and colleagues reported a method for releasing headspace gas in batch mode (Oh et al. 2009). Several researchers also reported higher undissociated organic acid concentration could inhibit cell growth and hydrogen production (Grupe and Gottschalk 1992; Kim et al. 2008a; Monot et al. 1984; Van Ginkel and Logan 2005).

2.2.3.2.6 Nitrogen, phosphate, metal ions

Ammonia nitrogen is important nitrogen source for fermentative hydrogen production. Phosphate is needed as a nutrient and can buffer the system. Appropriate C/N and C/P are necessary for fermentative hydrogen production by mixed culture (Hawkes et al. 2002; Wang and Wan 2009). Iron is an essential components of the hydrogenase enzyme, therefore trace levels of iron are required for fermentative hydrogen production (Wang and Wan 2009).

2.3 Electron shuttling compounds

Electron shuttling compounds are organic molecules that can cycle between oxidized and reduced forms and transfer electrons from lower redox potential electron donor to higher redox potential electron acceptor. Intracellular electron shuttles such as nicotinamide adenine dinucleotid (NAD^+), ubiquinone, ferredoxin and cytochromes play an essential role in electron transport chain of microorganisms (White 2000). Extracellular electron shuttles (EES) have been demonstrated for similar functions and applied to bioremediation (Lovley et al. 1998; Stams et al. 2006), biodegradation (Kwon and Finneran 2008), wastewater treatment (Watanabe et al. 2009) and microbial fuel cells (Aranda-Tamaura et al. 2007).

Soucaille's group investigated the influences of extracellular electron shuttles such as neutral

red and methyl viologen on carbon and electron flow in *Clostridium* fermentation (Girbal et al. 1995a; Girbal et al. 1995b; Peguin et al. 1994; Peguin and Soucaille 1995). For *Enterobacter aerogenes* in resting cells, batch cultures, and chemostat cultures, external NADH addition decreased hydrogen production (Zhang et al. 2009).

The application of extracellular electron shuttles for fermentative biohydrogen production was studied in Finneran's group, using the quinone-type electron shuttling compound anthraquinone-1, 6-disulfonate (AQDS) as a model. The amendment of reduced form (AH_2QDS) to improve fermentative hydrogen production was first observed in a cell suspension system (Hatch and Finneran 2008) and further demonstrated in *C. beijerinckii* growing cells for enhanced hydrogen molar yield and production rate as well as xylose utilization rate (Ye 2011; Ye et al. 2011). The increase was resulted from the metabolic pathway shift from butyric acid pathway to acetic acid pathway by affecting intracellular $NADH/NAD^+$ ratio and redistribution of reducing equivalents (Figure 2.2). More electrons flew from NADH to hydrogen via reduced ferredoxin instead of butyrate. AH_2QDS was also amended to the *Clostridium* fermentation using glucose, cellobiose, or mixed sugar with different glucose/xylose ratio as substrates and increased the hydrogen production rate (Ye et al. 2012a; Ye et al. 2012b).

2.4 Syntrophy

Syntrophic interactions that benefit co-existing organisms are prevalent in nature and have been applied to sludge digestion (Falony et al. 2009; Hatamoto et al. 2007), biodegradation (Falony et al. 2009) and bioremediation (Walker et al. 2009). Some researchers also suggested the application of syntrophic bacterial co-culture system to improve biofuel production. Aerobic *Bacillus* was used as an oxygen consumer to help create an anaerobic environment and generate useful enzymes for *Clostridium* fermentation (Chang et al. 2008). Similar strategy was used with

Enterobacter aerogenes (Yokoi et al. 1998). Co-culture of *R. sphaeroides* and *C. butyricum* in the phototrophic hydrogen production system has been applied, using the acetate and butyrate produced by *C. butyricum* as substrate to further produce hydrogen by *R. sphaeroides* (Fang et al. 2006). Syntrophy between *G. sulfurreducens* and *C. cellulolyticum* cultures were realized in microbial fuel cells (MFC) (Ren et al. 2008; Ren et al. 2007). In the MFC system, *C. cellulolyticum* fermented cellulose to acetate, ethanol and H_2. *G. sulfurreducens* oxidized these fermentation products and transferred electrons to the anode.

2.5 Geobacter metallireducens

The respiratory culture *Geobacter metallireducens* is a gram-negative, rod shaped, anaerobic bacteria (Lovley et al. 1993), which was first isolated from freshwater sediment. *G. metallireducens* uses the TCA pathway and can utilize short chain fatty acids, alcohols, and monoaromatic compounds, but not carbohydrate as electron donor, while using metals (*e.g.* iron, manganese and uranium) or AQDS as its electron acceptor (Ahrendt et al. 2007; Aklujkar et al. 2009; Boukhalfa et al. 2007; Champine et al. 2000; Cord-Ruwisch et al. 1998; Kane et al. 2002; Kwon and Finneran 2006; Kwon and Finneran 2008; Lovley et al. 1993; Wischgoll et al. 2005; Wolf et al. 2009b). It has been studied for the bioremediation of organic and metal contaminants in groundwater and participation in the carbon and nutrient cycles of aquatic sediments. Other EES such as juglone (Wolf et al. 2009b), lawsone (Lovley et al. 1998; Wolf et al. 2009b), fulvic acids (Fulton et al. 2004; Wolf et al. 2009b) and humic acids (Wolf et al. 2009b) have also been used as electron transfer mediators for bioremediation by *G. metallireducens*.

According to Figure 1.1, since volatile fatty acids are the major fermentative products besides H_2 during the acidogenic phase of *Clostridium* fermentation (Lengeler et al. 1999; Liu et al. 2006; Temudo et al. 2009; Vasconcelos et al. 1994), with the syntrophy of *C. beijerinckii*

and *G. metallireducens* in the presence of extracellular electron shuttles (*e.g.* AH_2QDS), *G. metallireducens* utilizes acetate to regenerate AH_2QDS, which then improves the fermentative biohydrogen production by *C. beijerinckii* (Ye et al. 2011).

2.6 Summary

Current research for biohydrogen production towards industrial application are focused on hydrogen molar yield, hydrogen production rate and substrate utilization extent. Previous research in our group found that *C. beijerinckii* fermentation with AH_2QDS amendment can significantly increase hydrogen yield and production rate as well as substrate utilization, which could provide a promising way for fermentative biohydrogen production. However, addition of the reduced form of the electron shuttle (AH_2QDS) is economically not feasible as it is unstable under aerobic conditions and its chemical regeneration is energy-consuming. Better methods to reduce AQDS and keep it in reduced form in the fermentation system need to be developed for the industrial application. Co-culture of *G. metallireducens* and *C. beijerinckii* is proposed to solve this problem and shorten the knowledge gap for the industrial scale biohydrogen production. This research will focus on this hypothesis.

Table 2.1 Comparison of different biohydrogen production processes

Process	Substrate	Advantages	Disadvantages
Biophotolysis	Water	H production directly from water and sunlight	O_2 limitation High intensity of light; Low photochemical efficiency
Photo fermentation	Organic acid	High yields; Utilization of organic wastes	Low light conversion efficiency
Dark fermentation	Carbohydrates	High production rate; all-day production without light; Various carbon sources; Easy operation with no O_2 limitation	Low yields

Table 2.2 Fermentative hydrogen yield using xylose, glucose or complex substrates

Substrate	Inoculum	Maximum H_2 yield	Reference
Xylose	*Clostridium acetobutylicum* CGS 5	0.73 mol/mol xylose	(Lo et al. 2008)
Xylose	Municipal sewage sludge	1.63 mol/mol xylose	(Wu et al. 2008b)
Xylose	*Thermoanaerobacterium* SCUT27 (Δldh mutant)	1.45 mol/mol xylose	(Li et al. 2010)
Glucose	*Clostridium acetobutylicum* ATCC824	1.08 mol/mol glucose	(Zhang et al. 2006a)
Glucose	*E. coli* BL-21 *hydA*$^+$	3.1 mol/mol glucose	(Chittibabu et al. 2006)
Glucose/Xylose mixture	*Thermoanaerobacterium* SCUT27 (*Δldh* mutant)	1.45 mol/mol xylose	(Li et al. 2010)
Food Wastewater	Waste activated sludge	4.71 mmol/g COD	(Wu and Lin 2004)

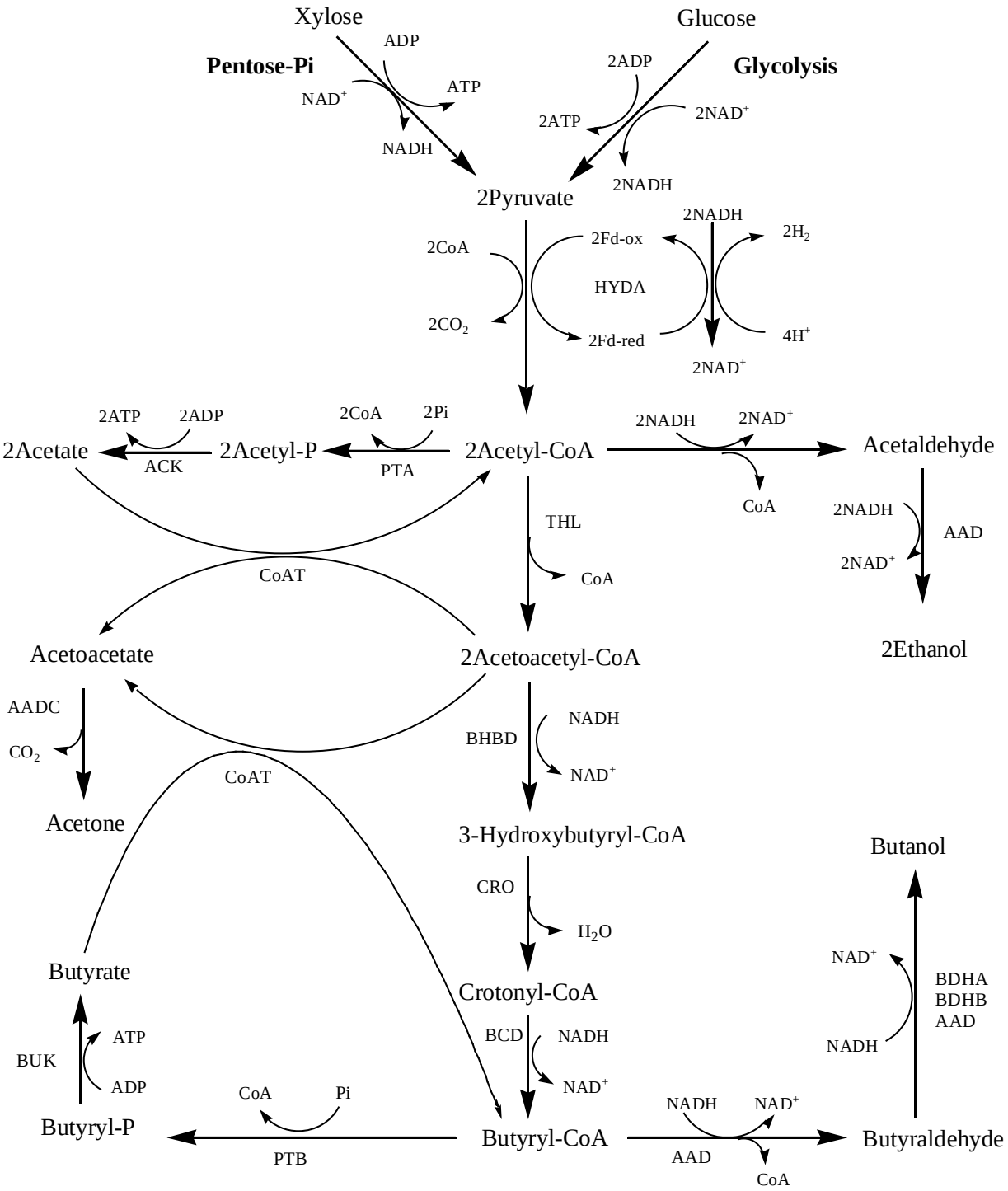

Figure 2.1 Adapted biochemical pathways of *Clostridium* fermentation (Lengeler et al. 1999; Liu et al. 2006; Temudo et al. 2009; Vasconcelos et al. 1994)

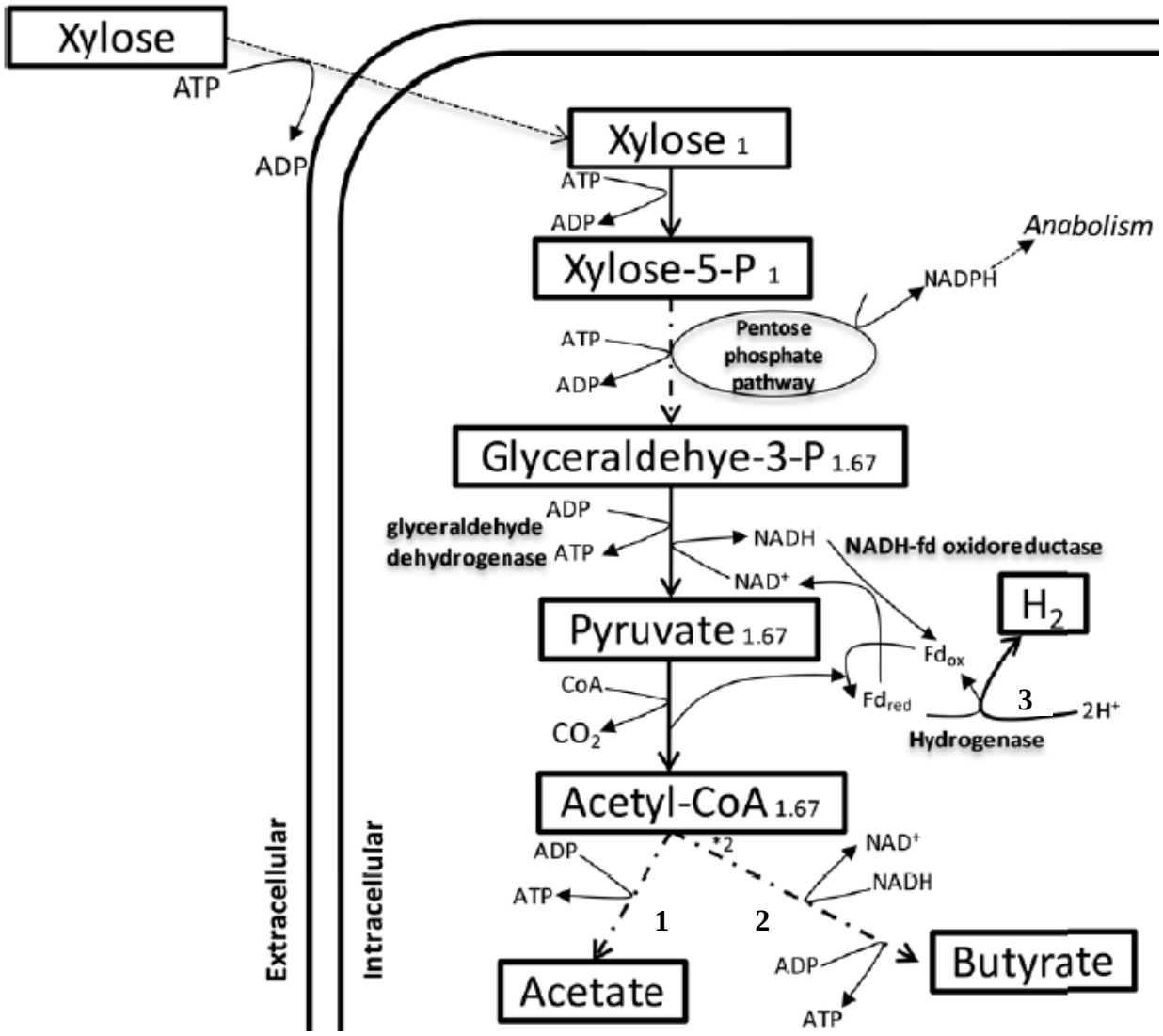

Figure 2.2 Modified *Clostridium* xylose fermentation pathway in the presence of AH_2QDS.

1. acetate production (non-redox reaction); 2. butyrate production (NADH consuming pathway); 3. Hydrogen production (NADH consuming pathway). AH_2QDS affects this fermentation by increasing 1, decreasing 2. Because 2 and 3 are competing for the electrons, addition of AH_2QDS increases the hydrogen production by increasing the electron flow from NADH (2) to Fd_{red} (3).

CHAPTER 3 INTERACTIONS BETWEEN *CLOSTRIDIUM BEIJERINCKII* AND *GEOBACTER METALLIREDUCENS* IN CO-CULTURE FERMENTATION WITH ANTHRAHYDROQUINONE-2, 6-DISULFONATE (AH_2QDS) FOR ENHANCED BIOHYDROGEN PRODUCTION FROM XYLOSE[1]

3.1 Abstract

To enhance biohydrogen production, *Clostridium beijerinckii* was co-cultured with *Geobacter metallireducens* in the presence of the reduced extracellular electron shuttle anthrahydroquinone-2, 6-disulfonate (AH_2QDS). In the co-culture system, increases of up to 52.3% for maximum cumulative hydrogen production, 38.4% for specific hydrogen production rate, 15.4% for substrate utilization rate, 39.0% for substrate utilization extent and 34.8% for hydrogen molar yield in co-culture fermentation were observed compared to a pure culture of *C. beijerinckii* without AH_2QDS. *G. metallireducens* grew in the co-culture system, resulting in a decrease in acetate concentration under co-culture conditions and a presumed regeneration of AH_2QDS from AQDS. These co-culture results demonstrate metabolic crosstalk between the fermentative bacterium *C. beijerinckii* and the respiratory bacterium *G. metallireducens* and suggest a strategy for industrial biohydrogen production.

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3.2 Introduction

Three key challenges for large-scale production of biofuels such as hydrogen are increasing i) the hydrogen production rate, ii) the hydrogen molar yield, and iii) the extent of substrate utilization. These issues are particularly critical when using xylose as a substrate compared to relatively efficient utilization of glucose (Bartacek et al. 2007; Kim et al. 2010; Kuhad et al. 2011; Sarkar et al. 2012). Xylose, as one of the two major hydrolysates from lignocellulosic biomass pretreatment and enzymatic hydrolysis (Weber et al. 2010), is the second most abundant potential resource for fermentative biofuel production (de Vrije et al. 2002; Maintinguer et al. 2011; Wohlbach et al. 2011). However, the xylose-dependent biohydrogen molar yield and production rate, as well as substrate utilization, are generally low (Li et al. 2010; Lo et al. 2008; Long et al. 2010; Maintinguer et al. 2011). To improve these parameters, extracellular electron shuttles (EES) and a co-culturing approach are applied here. EES have previously been applied to biofuel production (Hatch and Finneran 2008; Ye et al. 2012a; Ye et al. 2011), bioremediation (Kwon and Finneran 2008; Lovley et al. 1998; Stams et al. 2006), wastewater treatment (Watanabe et al. 2009), microbial fuel cells (Aranda-Tamaura et al. 2007), and fermentation (Girbal et al. 1995a; Girbal et al. 1995b; Peguin et al. 1994; Peguin and Soucaille 1995; Zhang et al. 2009).

Clostridium beijerinckii is a robust biohydrogen-generating fermenter, based on its specific hydrogen production rate and yield (Benemann 1996; Jeong et al. 2008). When using the degenerated strain of *C.beijerinckii*, the major soluble fermentation products along with hydrogen were volatile fatty acids including acetate and butyrate. Furthermore, Hatch and Finneran (2008) found that the addition of the reduced extracellular electron shuttle anthrahydroquinone-2, 6-disulfonate (AH_2QDS) increased cumulative biohydrogen production by *C. beijerinckii*. Applying this strategy to growing cells, Ye et al. (2012a; 2011) found the

amendment of AH_2QDS to *C. beijerinckii* growing cells enhanced the hydrogen molar yield, the hydrogen production rate, and the extent of xylose utilization. These improvements were due to a metabolic shift from the butyric acid pathway to the acetic acid pathway, resulting from changes in the intracellular $NADH/NAD^+$ ratio in the presence of AH_2QDS (Ye et al. 2011). Adding AH_2QDS could therefore be an effective strategy to improve biohydrogen production.

However, addition of AH_2QDS is not economically feasible. In reduced form it is unstable under aerobic conditions. It is also oxidized to AQDS during the fermentation. For application in a continuous reactor, efficient processes are needed to regenerate AH_2QDS. In the current work, we investigated overcoming this obstacle through biological in-situ regeneration of AH_2QDS via co-culture with *Geobacter metallireducens* (Figure 1.1). *G. metallireducens* can utilize AQDS as electron acceptor, regenerating AH_2QDS (Aklujkar et al. 2009; Kwon and Finneran 2006; Kwon and Finneran 2008; Lovley et al. 1993; Wolf et al. 2009a). Furthermore, *G. metallireducens* utilizes short chain fatty acids (*e.g.* acetate), alcohols and monoaromatic compounds as electron donors (Ahrendt et al. 2007; Boukhalfa et al. 2007; Champine et al. 2000; Cord-Ruwisch et al. 1998; Kane et al. 2002; Wischgoll et al. 2005). Since volatile fatty acids are the major soluble fermentation products during the acidogenic phase of clostridial fermentations (Liu et al. 2006; Temudo et al. 2009; Vasconcelos et al. 1994), no additional substrate for *G. metallireducens* should be required. Finally, *G. metallireducens* does not utilize carbohydrates or H_2, so it will not compete for the substrate or consume our target product in the proposed co-culture system (Lovley et al. 1993).

Co-cultures and syntrophic interactions have been applied previously, including for sludge digestion (Falony et al. 2009; Hatamoto et al. 2007) and biodegradation (Falony et al. 2009; Walker et al. 2009). Some researchers have also suggested the application of syntrophic bacterial

co-culture systems to improve biofuel production (Chen 2011), for example to consume oxygen in an aerobic/anaerobic system (Chang et al. 2008; Yokoi et al. 1998), to remove fermentation products in phototrophic biofuel production system (Fang et al. 2006), to stimulate the conversion rate of substrate (Chou et al. 2011), or to produce essential nutrients (He et al. 2011). However, co-culture for EES regeneration, such as that proposed here, has not to our knowledge been applied previously, particularly in the context of biofuel production. Electron transfer between species is however possible based on the example of 2-amino-3-carboxy-1, 4-naphtoquinone, which has been extracted from freeze-dried cells of *Propionibacterium freundenreichii* and used to stimulate the growth of *Bifidobacterium longum* (Hernandez and Newman 2001; Yamazaki et al. 1999).

The objective of the current study was to investigate the biological in-situ regeneration of AH_2QDS and biohydrogen production using co-culture system of *C. beijerinckii* and *G. metallireducens* with AH_2QDS. The motivation was to identify more economically feasible ways to apply the strategy of AH_2QDS addition to *C. beijerinckii* (Ye et al. 2011) for enhanced biohydrogen production. This was accomplished by measuring hydrogen and volatile acid production, xylose utilization, and culture growth through Q-PCR. The working model (Figure 1.1) includes *C. beijerinckii* fermenting xylose, with production of H_2, acetate, and butyrate and oxidation of AH_2QDS to AQDS. Concurrently, *G. metallireducens* oxidizes acetate and butyrate to regenerate AH_2QDS, which increases H_2 production by *C. beijerinckii*.

3.3 Materials and Methods

3.3.1 Culture Maintenance

Clostridium beijerinckii NCIMB 8052 was obtained from TetraVitae Bioscience, Inc,

(Champaign, IL), and *Geobacter metallireducens* GS-15 was obtained from Dr. Lovely (University of Massachusetts, Amherst, Massachusetts). *C. beijerinckii* was transferred to modified P2 medium (Baer et al. 1987) with 10mM glucose every week to limit solvent production and incubated at 37°C. *G. metallireducens* was maintained in fresh water medium (Lovley et al. 1993) at 30°C with 20 mM acetate as the electron donor and 5 mM AQDS instead of soluble Fe (III) citrate as the electron acceptor. All media were sonicated and flushed with N_2 to achieve anaerobic conditions prior to autoclaving at 121°C for 20 minutes.

3.3.2 Chemicals

Anthraquinone-2, 6-disulfonate (AQDS) was purchased from the AKSCI (Union City, CA, USA). To generate chemically reduced AH_2QDS, 5 mM AQDS solution with 30 mM bicarbonate buffer was bubbled with 80:20 H_2:CO_2 for at least 1 hour in the presence of 100 g/L palladium-coated aluminum catalyst (Sigma, St. Louis, MO, USA) and then incubated at 30°C overnight. AH_2QDS stock solution was then bubbled with H_2-free N_2 and filtered twice with 0.2 μm filter (PALL Acrodisc® syringe filter) into a sterile, evacuated pressure tube.

3.3.3 Batch experiments

Batch tests with 10 mL of culture in 26mL anaerobic tubes were performed at 30°C in the dark. Modified fresh water medium was developed for the co-culture batch experiments and consisted of phosphate buffer (1 g/L K_2HPO_4 and 1 g/L KH_2PO_4) instead of the original bicarbonate buffer. The initial pH is around 6.5 and the final pH values were similar irrespective of hydroquinone or/and *G. metallireducens* amendment. Eight fermentation conditions were carried out, including three different co-culture conditions (containing no additions, *G. metallireducens* spent medium, or *G. metallireducens* fresh medium), three single culture *C. beijerinckii* conditions (containing

no additions, *G. metallireducens* spent medium, or chemically reduced AH_2QDS), a *G. metallireducens* single culture with residual AH_2QDS from inoculum, and an abiotic control. The *G. metallireducens* spent medium was prepared by filtering out the cells with 0.2 μm filter (PALL Acrodisc® syringe filter) and contained about 5mM biologically reduced AH_2QDS. For the conditions with spent medium, 10% spent medium was added for a final concentration of around 0.5mM AH_2QDS. For the conditions without spent medium, chemically reduced AH_2QDS was amended to achieve similar initial AH_2QDS concentrations. 2 g/L xylose was provided as the carbon source.

The *G. metallireducens* inoculum was grown in fresh water medium with 50mM soluble Fe (III) citrate as electron acceptor, concentrated and washed as previously described for preparation of resting cell suspensions (Kwon and Finneran 2006), while the *C. beijerinckii* inoculum was grown in modified P2 medium for 1 day as described in culture maintenance and used directly. The inoculation ratio was 1% for concentrated and washed *G. metallireducens* inoculum and 3% for *C. beijerinckii* inoculum. All experiments were run in triplicate. Headspace and aqueous samples were collected every 4 hours. Headspace samples were analyzed for H_2 concentration immediately, and the liquid samples were filtered and stored at 4°C until analysis.

3.3.4 Analytical Techniques

Headspace hydrogen gas was monitored by gas chromatograph (Shimadzu GC-14A) equipped with a thermo conductive detector (TCD, SRI instrument Model 110). Total hydrogen production was calculated according to the headspace hydrogen concentration and corresponding liquid hydrogen concentration using Henry's law. Filtered aqueous samples were analyzed for organic acids using HPLC (Dionex Summit) with Transgenomic® Organic Acid column and for acetone, butanol and ethanol using GC-FID (Shimadzu-GC2014) with DB-FFAP capillary column (Ye et

al. 2011). Xylose was measured by HPLC (Shimadzu) with a refractive index detector (Waters, Milford, MA, USA) and Shodex (New York, NY, USA) sugar SP0810 column with ionic form H^+/CO_3^- deashing guard column using nanopure water as mobile phase with a flow rate of 0.6 ml/min at 85 °C. The concentration of AH_2QDS was determined by the Ferrozine assay as described previously (Kwon and Finneran 2006; Lovley et al. 1996; Lovley and Phillips 1987).

3.3.5 Molecular Techniques

For quantification of growth of the two microorganisms in co-culture, biomass samples were taken at the initial and final time points in batch tests and analyzed using Q-PCR. Genomic DNA was extracted using the Fast DNA Spin Kit (Qbiogene, Carlsbad, CA, USA). Primers Chis150f and ClostIr were used to quantify the 16S rDNA of *C. beijerinckii* (Hung et al. 2008), while Geo494F/Geo825R (Holmes et al. 2002) were used for *G. metallireducens*. For every 25 µL Q-PCR master mix, 3 µL DNA (approximately 450 ng) was used. Q-PCR was performed on a Bio-Rad (Hercules, CA, USA) CFX96 TM Real time system using SYBR Green chemistry. The amplification conditions for *C. beijerinckii* were 95°C for 3 min, 30 cycles of 95°C for 45 s, 57°C for 45 s, and 72°C for 2 min, followed by 72°C for 3 min for extension. For a positive control, PCR products from the *C. beijerinckii* pure culture and primers 10F/1492R (Esikova et al. 2002) were used as the DNA template. Two negative controls were performed, one with no DNA and one with PCR products from the *G. metallireducens* pure culture and primers 338F/907R (Holmes et al. 2002). The amplification conditions for *G. metallireducens* were 50°C for 2 min, 95°C for 10 min, followed by 30 cycles of 95°C for 15 s and 56°C for 60 s. In this case PCR products of *G. metallireducens* pure culture provided the positive control while PCR products of *C. beijerinckii* pure culture and no DNA were used as negative controls. Triplicate Q-PCR was performed for each DNA extraction. The standard deviation was less than 20% for

these triplicate Q-PCR experiments.

3.3.6 Kinetic modeling for hydrogen production, product formation and substrate utilization

A modified Gompertz equation (Lay et al. 1998) was used to simulate the cumulative hydrogen production. The model was applied to each batch experiment to obtain the maximum hydrogen production $P_{max,i}$, product formation rate R_i and lag phase λ_i, where i represents hydrogen. The same equation was also applied to fit the substrate utilization.

$$P_i = P_{\max,i} \times \exp\{-\exp[\frac{\exp(1) \times R_i}{P_{\max,i}}(\lambda_i - t) + 1]\}$$

(3.1)

$P_{hydrogen}$ and $P_{max,hydrogen}$ are in the unit of μmol; $R_{hydrogen}$ is in the unit of μmol/h; $P_{(max),\ xylose}$ are in the unit of mM; R_{xylose} are in the unit of mM/h; λ_i is in the unit of hour for all the products.

3.3.7 Statistical analysis

Residual plots (Q'Q plots, correlogram) were used to evaluate the assumption of normal distributed independent errors and therefore that the estimated parameter uncertainty is reliable. Based on the estimated parameters and their standard deviations (Table 3.1) it was tested if the parameters differ statistically significant for the different conditions and corresponding p-values were calculated (Table 3.2).

3.4 Results

3.4.1 H_2 production and substrate utilization

Co-cultures of *C. beijerinckii* and *G. metallireducens* with AH_2QDS showed improved hydrogen

production as compared to single cultures of *C. beijerinckii* (Figure 3.1 and Table 3.1). To distinguish whether this improvement in hydrogen production required *G. metallireducens* cells or was due to media components or residual metabolic products in the *G. metallireducens* inoculum, a variety of conditions were compared in batch tests, including addition of fresh or spent media. The hydrogen production profiles fell into three clusters: *C. beijerinckii* alone, *C. beijerinckii* with AH_2QDS, and co-cultures of *C. beijerinckii* and *G. metallireducens* (Figure 3.1 and Table 3.1). The *C. beijerinckii* pure culture had the longest lag phase and the lowest maximum hydrogen production, specific hydrogen production rate, and substrate utilization rate. Adding AH_2QDS to the *C. beijerinckii* pure culture shortened the lag phase and increased the maximum hydrogen production, the specific hydrogen production rate, and the substrate utilization rate compared to *C. beijerinckii* pure culture, and this was true whether the AH_2QDS was chemically (*C. b.* + chemically reduced AH_2QDS in Figure 3.1 and Table 3.1) or biologically reduced (*C. b.* + *G. m.* spent media in Figure 3.1 and Table 3.1). The presence of both AH_2QDS and *G. metallireducens* (all co-culture conditions) provided an additional significant improvement in the hydrogen production and substrate utilization (p-values < 0.1 in Table 3.2), regardless of whether fresh or spent media was added. Compared to *C. beijerinckii* alone, co-culture fermentation achieved up to a 52.3% increase in the maximum cumulative hydrogen production, a 38.4% increase in the specific hydrogen production rate, and a 15.4% increase in the substrate utilization rate, while shortening the lag phase significantly, from 22 h to as little as 10 h.

Higher maximum hydrogen production may result from an increase in hydrogen yield, an increase in substrate utilization, or both. Compared to *C. beijerinckii* alone, both the co-culture and *C. beijerinckii* with AH_2QDS obtained higher yields (Figure 3.2). However, there was no

significant difference between the co-culture and *C. beijerinckii* plus AH_2QDS, indicating that the increase in hydrogen yield was mainly due to the presence of AH_2QDS. AH_2QDS also increases the specific substrate utilization, from 44% to about 60%. However, in this case adding *G. metallireducens* further increased the substrate utilization, to around 70%. The co-culture benefits are therefore a combination of the effects of AH_2QDS and *G. metallireducens*, with the co-culture acting primarily by enhancing substrate utilization.

3.4.2 Growth of *G. metallireducens* and *C. beijerinckii*

To assess growth of each organism in co-culture, the difference between initial and final culture density of *G. metallireducens* and *C. beijerinckii* was determined by Q-PCR (Figure 3.3). In *G. metallireducens* pure culture control, the abundance of *G. metallireducens* did not change, which was expected since this organism cannot grow on xylose. In the co-culture system, the final *G. metallireducens* abundance was 10 to 50 times higher than the initial values, for a statistically significant difference at a 90% confidence level (Table 3.2).

Comparing the *C. beijerinckii* abundance at the end of the batch tests, the pure culture without AH_2QDS (2.94×10^9 copy number/mL culture) was significantly lower than either the pure culture with AH_2QDS (7.32×10^9 ~ 1.74×10^{10} copy number/mL culture) or the co-culture system (1.36×10^{10} ~ 2.37×10^{10} copy number/mL culture). There was no significant difference between the *C. beijerinckii* with AH_2QDS and the co-culture system (Table 3.2).

3.4.3 Acetate utilization by *G. metallireducens*

C. beijerinckii NCIMB 8052 was degenerated to block solvent production, and measurements confirmed that ethanol, butanol and acetone concentrations were below their detection limits. Therefore, acetate and butyrate are the major soluble fermentation products. The co-culture

conceptual model (Figure 1.1) predicts that the addition of *G. metallireducens* will prevent or reduce the accumulation of acetate and butyrate. The acetate measurements supported this prediction (Figure 3.4). The initial acetate concentrations varied due to residual acetate in the *G. metallireducens* spent media, which typically contained about 2.5 mM acetate, and in the *G. metallireducens* inoculum, which contained about 0.6 mM acetate. In the *C. beijerinckii* pure culture system, acetate increased in the exponential phase and then remained relatively stable in stationary phase, paralleling the fermentative hydrogen production profile. Cultures with AH_2QDS (chemically or biologically reduced) showed a shorter lag phase before beginning acetate production. In the co-culture system, the acetate concentration also increased at the beginning, but then decreases, presumably due to utilization by *G. metallireducens*. There is no substantial decrease of butyrate in co-culture system (Figure 3.5).

3.5 Discussion

3.5.1 Benefits of co-culture fermentation for hydrogen production

The results presented here demonstrate that co-cultures of *C. beijerinckii* and *G. metallireducens* exhibit improved xylose utilization, suggesting a more practical method to integrate extracellular electron shuttles into fermentative biohydrogen production. Compared to *C. beijerinckii* alone, addition of AH_2QDS resulted in significantly higher maximum hydrogen production (from 130.1 to 174.3 ~ 184.3 μmole), substrate utilization rates (0.17 to 0.29 ~ 0.30 mM/h), and extent of substrate utilization (45% to around 60%) (Table 3.1, Figure 3.2). This phenomenon has been reported previously (Hatch and Finneran 2008; Ye et al. 2011) and is attributed to a metabolic shift from the butyric acid pathway to the acetic acid pathway (Ye et al. 2011).

The current work demonstrates in addition that i) biologically reduced AH_2QDS functions

similarly to chemically reduced AH_2QDS, and ii) co-culture with *G. metallireducens* can further improve hydrogen production. The substitution of biologically reduced AH_2QDS for chemically reduced AH_2QDS is supported by the similar kinetic results, hydrogen molar yield, and substrate utilization under each of those conditions (Table 3.1). Co-culture with *G. metallireducens* resulted in further improvements, including statistically significant increases in maximum hydrogen production (from 184.3 µmole to 223.1 µmole), substrate utilization rate (from 0.3028 to 0.3766 mM/h) and specific substrate utilization (from 63% to 77%) as compared to *C. beijerinckii* with AH_2QDS. There was no significant difference in hydrogen production among co-cultures amended with spent or fresh *G. metallireducens* media, which suggests that this improvement was due to *G. metallireducens* activity. While the increase in hydrogen production upon addition of AH_2QDS was due to increases in both hydrogen molar yield and substrate utilization, the co-culture resulted primarily in improved substrate utilization.

3.5.2 Interactions between *G. metallireducens* and *C. beijerinckii*

These experiments support a model in which *C. beijerinckii* produces more hydrogen, acetate and butyrate in the presence of AH_2QDS and *G. metallireducens* uses the acetate while regenerating AH_2QDS (Figure 1.1). We propose that syntrophic or cross-feeding interactions are occurring in these co-culture fermentations. This proposal is supported by the *G. metallireducens* Q-PCR data, which shows growth in the co-culture system and not in single culture (Figure 3.4). *G. metallireducens* requires the acetate produced by *C. beijerinckii* for growth. The effects on *C. beijerinckii* are evident in our experiments as a change in activity, with greater cumulative H_2 production and substrate utilization and acetate consumption in the co-culture system, but the addition of *G. metallireducens* did not result in an increase in *C. beijerinckii* abundance, so mutual cross-feeding has not been demonstrated. It is possible that an effect on growth of *C.*

beijerinckii might be observed in longer term or continuous experiments.

3.5.3 Industrial relevance

Co-culture fermentation with AH_2QDS suggests a more practical strategy to improve biohydrogen production by providing both an economical method for regenerating the reduced extracellular electron shuttle AH_2QDS and a means to diminish product inhibition. Low pH due to the accumulation of volatile organic acids (*e.g.* acetic acid and butyric acid) is known to trigger a shift from the acidogenic phase to the solventogenic phase and lower hydrogen production in *Clostridium* fermentation (Gottschal and Morris 1981; Gottwald and Gottschalk 1985; Grupe and Gottschalk 1992; Monot et al. 1984; Riebeling et al. 1975; Terracciano and Kashket 1986; Van Ginkel and Logan 2005). *G. metallireducens* in the co-culture can utilize acetate and regenerate AH_2QDS in-situ, opening the possibility of a continuous production mode. In this study, we used xylose as substrate and the model EES compound AH_2QDS, but for an economically-viable process less expensive substrates and EES will be required, such as hydrolysates from lignocellulosic biomass pretreatment and extracellular electron shuttles like humic acids.

3.6 Conclusion

In this research, we demonstrate interactions between *C. beijerinckii* and *G. metallireducens* in the presence of extracellular electron shuttle AH_2QDS, resulting in increased hydrogen production and substrate utilization. This co-culture fermentation allows biological in-situ regeneration of the AH_2QDS and reduces accumulation of acetate during xylose fermentation, providing a novel strategy to improve biohydrogen production. Application of this co-culture system to practical substrates and electron shuttles should provide an economical implementation

of this strategy.

Acknowledgments We thank Hans Blaschek and Steven Stoddard of TetraVitae Biosciences for *Clostridium beijerinckii* spores, Derek R. Lovley of the University of Massachusetts for *Geobacter metallireducens*, and Mr. Andreas Scheidegger of Eawag for support of statistical analysis. This work was supported by National Science Foundation grant no. 0756054.

Table 3.1 Summary of kinetic parameters in the modified Gompertz model for fermentative hydrogen production at different experimental conditions using xylose as substrate

Condition	Lag phase, λ_i (h)	Maximum H_2 production, $P_{max,hydrogen}$ (μmole)	Specific H_2 production rate, $R_{hydrogen}/V$ (mmol/L/h)	Substrate utilization rate, $R_{substrate}$ (mM/h)
Co-culture + *G.m.* spent media	10.39±2.24	198.2±10.9	0.93±0.22	0.38±0.06
Co-culture + *G.m.* fresh media	13.16±0.53	223.1±2.9	0.86±0.04	0.33±0.04
Co-culture	13.43±0.51	212.8±2.7	0.82±0.04	0.36±0.12
C.b. + *G.m.* spent media	20.23±0.69	174.3±4.2	0.80±0.05	0.30±0.05
C.b. + chemically reduced AH_2QDS	19.23±0.76	184.3±3.7	0.84±0.07	0.29±0.06
C.b. alone	22.35±1.67	130.1±6.1	0.67±0.14	0.17±0.08

The "±" in the table stands for the standard error (residuals) for the estimation of the parameters. According to the statistical analysis, the model parameters are valid since (a) their residuals are normal distributed; (b) the variance is more or less constant; and (c) the residuals are independent

Table 3.2 Probability over the joint distribution between different conditions for the kinetic parameters

Parameter	Conditions to compare	p-value	Co-culture is significant greater than pure culture (90% confidence level)
Maximum hydrogen production	Co-culture vs. *C. b.* + AH_2QDS	$2.4\times e^{-04}$	Yes
Lag phase	Co-culture vs. *C. b.* + AH_2QDS	$1.4\times e^{-05}$	Yes
Substrate utilization rate	Co-culture vs. *C. b.* + AH_2QDS	$9.3\times e^{-02}$	Yes
Specific substrate utilization extent	Co-culture vs. *C. b.* + AH_2QDS	$0.6\times e^{-03}$	Yes
Cell density of *C. beijerinckii*	Co-culture vs. *C. b.* + AH_2QDS	$6.7\times e^{-01}$	No
Cell density of *G. metallireducens*	Co-culture vs. *G.m.* alone	$7.6\times e^{-03}$	Yes

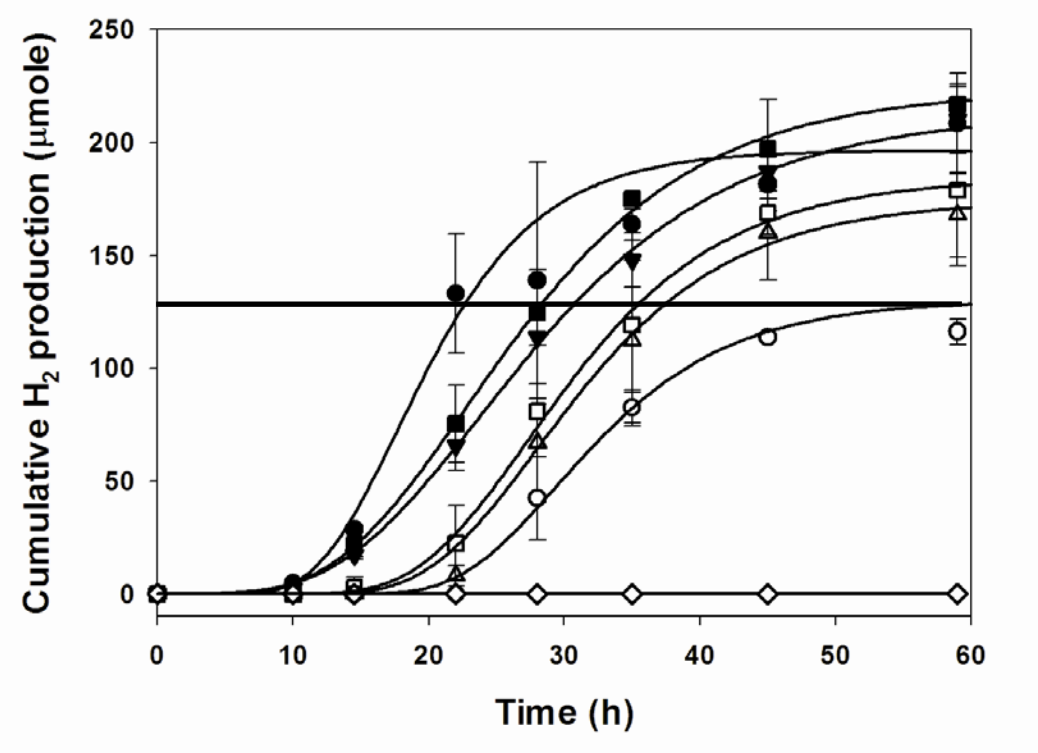

Figure 3.1 Experimental data and kinetic model fits of cumulative hydrogen production versus time. Data points are the means of triplicate cultures. Error bars refer to one standard deviation. Lines are the fitted curves of the Modified Gompertz equation (Eq 3.1). The horizontal line marks the maximum hydrogen production by *C. beijerinckii* without AH_2QDS or *G. metallireducens*. *C.b.* stands for *Clostridium beijerinckii* and *G.m.* stands for *Geobacter metallireducens*. ●: Co-culture + *G.m.* spent medium; ■: Co-culture + *G.m.* fresh medium; ▼: Co-culture; ▵: *C.b.* + *G.m.* spent medium; □: *C.b.* + chemically reduced AH_2QDS; ○: *C.b.* alone; ◆: *G.m.* alone; –◇–: Abiotic control.

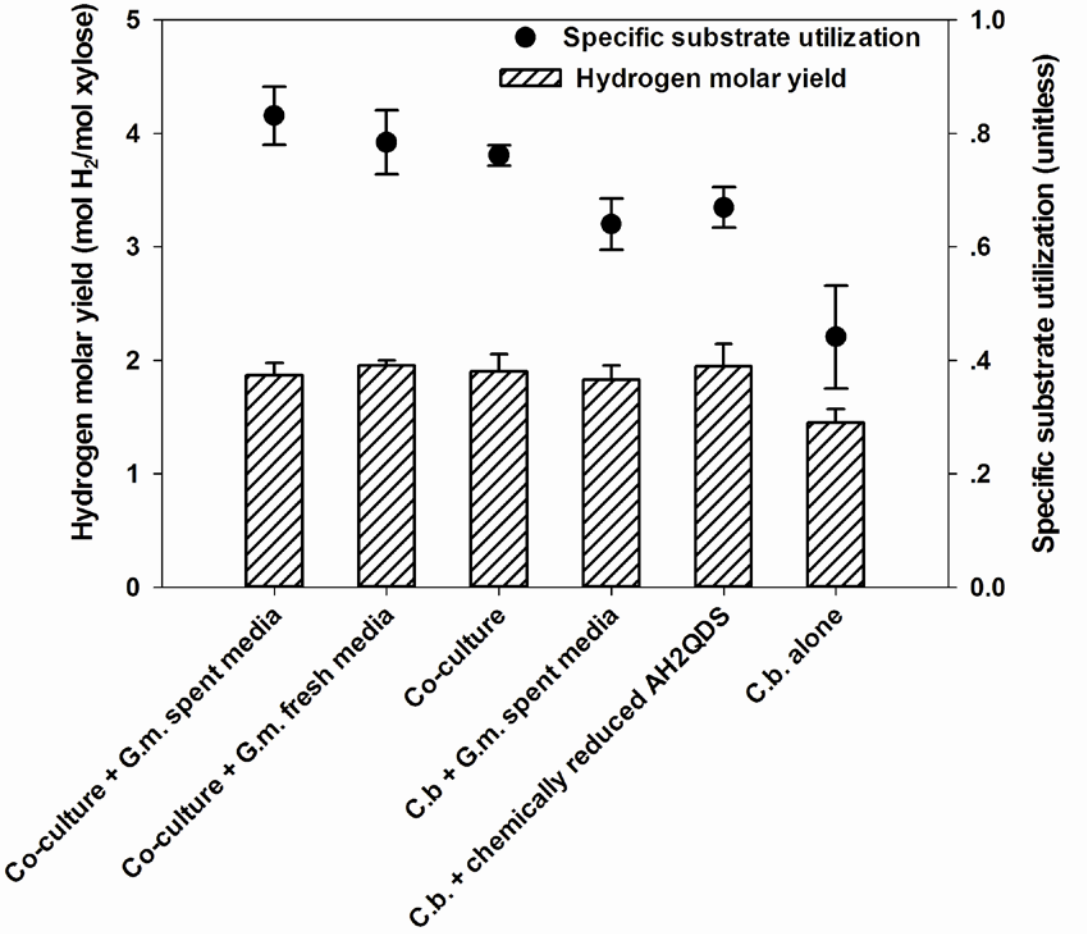

Figure 3.2 Hydrogen molar yield and specific substrate (xylose) utilization. (Specific xylose utilization refers to the moles of xylose consumed versus the initial moles of xylose)

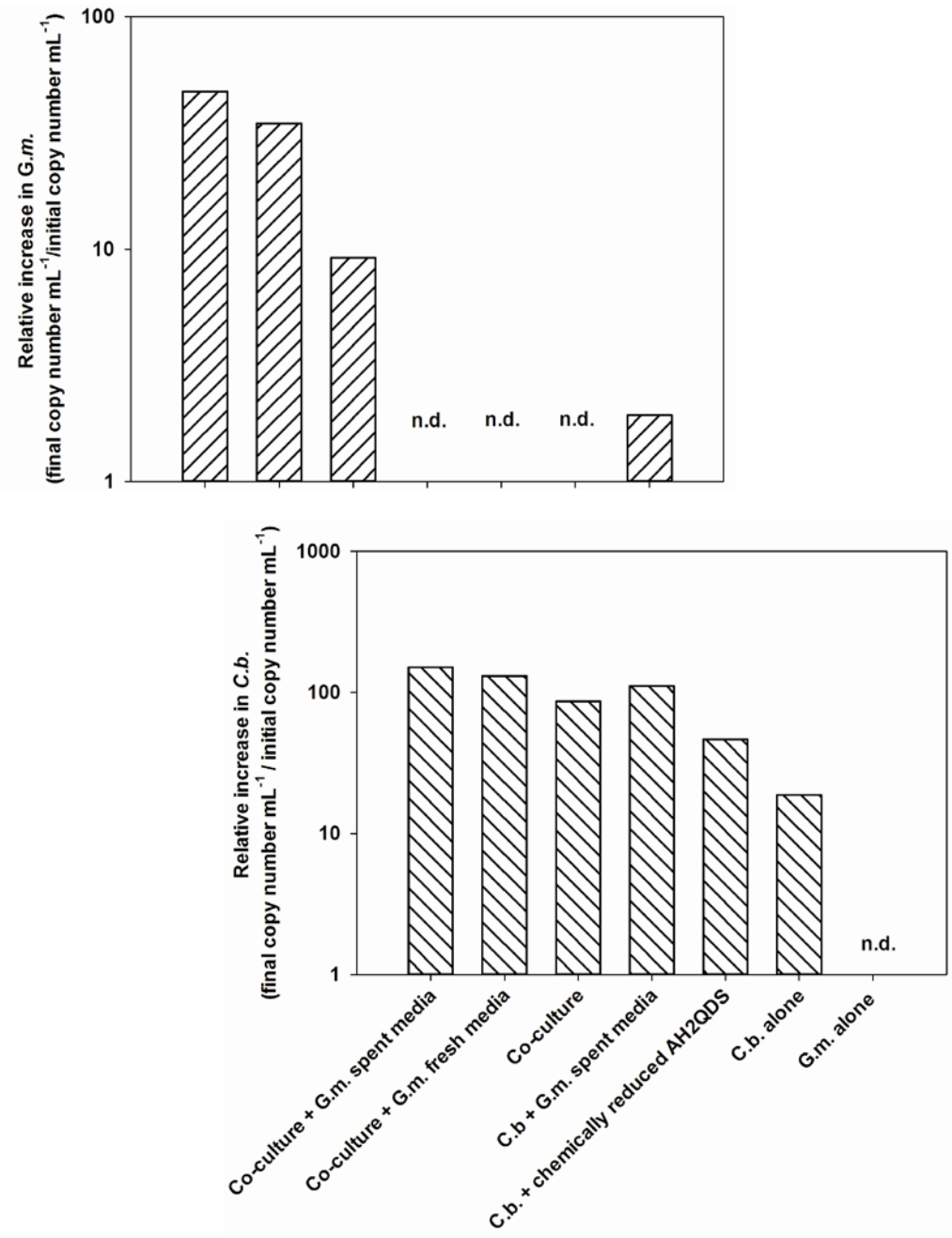

Figure 3.3 Change in abundance of *G. metallireducens* and *C. beijerinckii* during batch experiments. There were significant increases of *G. metallireducens* abundance in all co-culture conditions compared to *G. metallireducens* alone (with residual AH_2QDS from inoculum), but no significant difference within the co-culture conditions. *C. beijerinckii* abundance increased significantly in either the pure culture with AH_2QDS (7.32×10^{09} ~1.74×10^{10} copy number/mL culture) or the co-culture system (1.36×10^{10} ~2.37×10^{10} copy number/mL culture) compared to pure culture without AH_2QDS, but there was no significant difference between the *C. beijerinckii* with AH_2QDS and the co-culture system. Data points are the means of triplicate cultures and sampling values. Standard deviations averaged about 20% of the copy number. n.d., not determined.

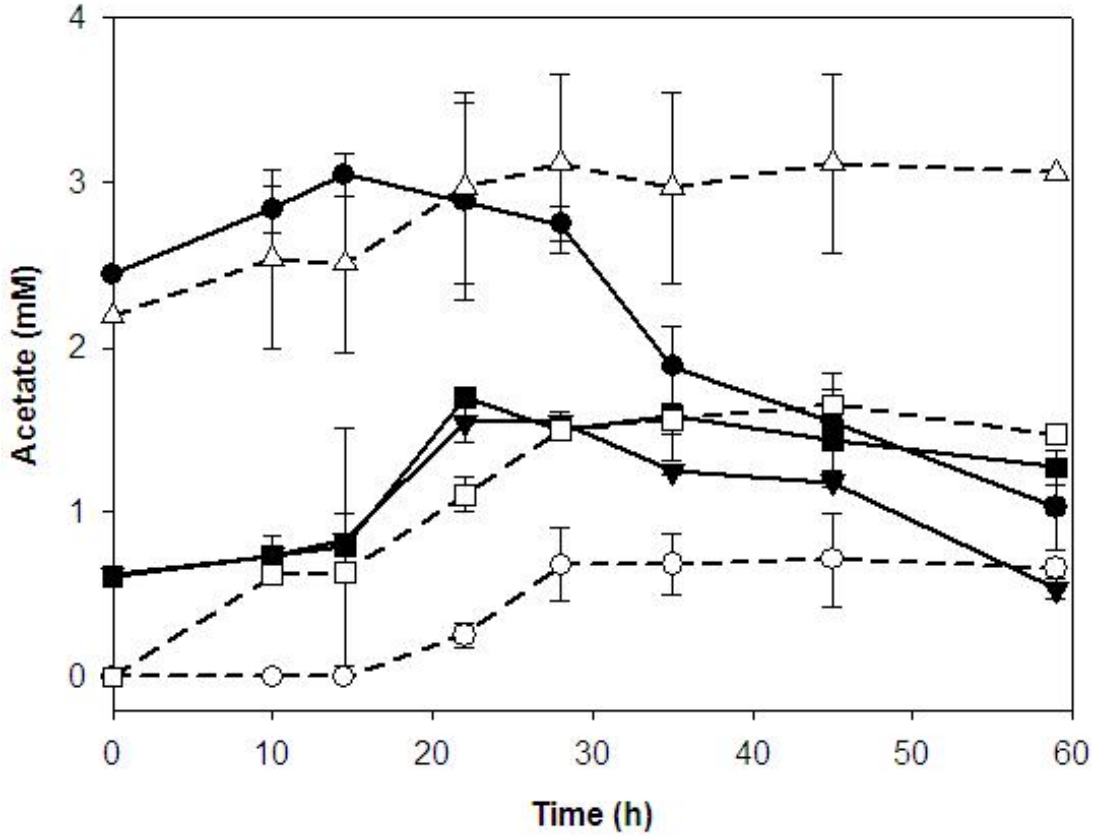

Figure 3.4 Profile of acetate (one of the fermentation products of *C. beijerinckii*) versus time. Data points are the means of triplicate sampling values. Bars refer to one standard deviation. Solid lines with solid symbols for all the co-culture conditions and dashed lines with hollow symbols for the single culture (with or without AH_2QDS). ●: Co-culture + *G.m.* spent media; ■: Co-culture + *G.m.* fresh media; ▼: Co-culture; ∆: *C.b.* + *G.m.* spent media; □: *C.b.* + chemically reduced AH_2QDS; ○: *C.b.* alone.

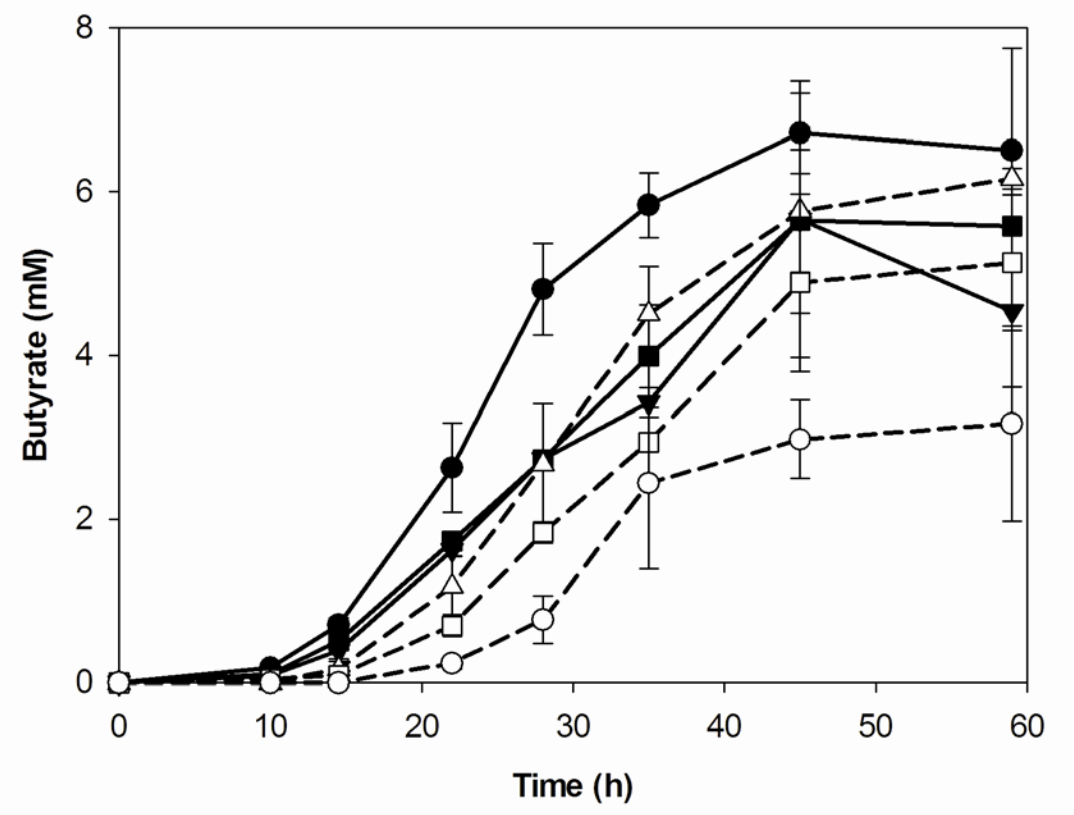

Figure 3.5 Profile of butyrate (one of the fermentation products of *C. beijerinckii*) versus time. Data points are the means of triplicate sampling values. Bars refer to one standard deviation. Solid lines with solid symbols for all the co-culture conditions and dashed lines with hollow symbols for the single culture (with or without AH_2QDS). ●: Co-culture + *G.m.* spent media; ■: Co-culture + *G.m.* fresh media; ▼: Co-culture; ∆: *C.b.* + *G.m.* spent media; □: *C.b.* + chemically reduced AH_2QDS; ○: *C.b.* alone.

CHAPTER 4 LIGNOCELLULOSIC HYDROLYSATES AND ALTERNATIVE ELECTRON SHUTTLES FOR H_2 PRODUCTION USING CO-CULTURE FERMENTATION WITH *CLOSTRIDIUM BEIJERINCKII* AND *GEOBACTER METALLIREDUCENS*[2]

4.1 Abstract

A co-culture of *Clostridium beijerinckii* and *Geobacter metallireducens* with AH_2QDS produced hydrogen from lignocellulosic hydrolysates (biomass of *Miscanthus x giganteus* prepared by hydrothermal treatment with dilute acids). This co-culture system enhanced hydrogen production from lignocellulosic hydrolysates by improving substrate utilization and diminishing acetate accumulation, despite the presence of fermentation inhibitors in the hydrolysates. The improvements were greater for xylose-rich hydrolysates. The increase in maximum cumulative hydrogen production for hydrolysates with glucose:xylose ratios of 1:0.2, 1:1 and 1:10 was 0, 22% and 11%, respectively. Alternative extracellular electron shuttles (EES), including indigo dye, juglone, lawsone, fulvic acids and humic acids, were able to substitute for AH_2QDS, improving hydrogen production in the co-culture system using xylose as model substrate. Increased utilization of xylose-rich hydrolysates and substitution of alternative EES make the co-culture with EES system a more attractive strategy for industrial biohydrogen production.

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[2] This chapter is currently under review: Zhang, X., Ye, X., Finneran, K.T., Zilles, J. Guo, B. and Morgenroth, E. (2012) Lignocellulosic hydrolysates and alternative electron shuttles for H_2 production using co-culture fermentation with *Clostridium beijerinckii* and *Geobacter metallireducens*. *International Journal of Hydrogen Energy* submitted.

4.2 Introduction

Dark fermentation with *Clostridium* is a promising strategy for biohydrogen production (Hallenbeck and Benemann 2002; Turner et al. 2008). A major barrier for industrial application of fermentative biohydrogen production is the high cost when compared to thermal/chemical hydrogen production (Bartacek et al. 2007; Hallenbeck and Benemann 2002; Turner et al. 2008). Efficient utilization of hydrolysates from lignocellulosic biomass could be a solution to these obstacles by providing inexpensive, abundant and varied substrates for biohydrogen production (de Vrije et al. 2002; Kapdan and Kargi 2006; Sarkar et al. 2012; Saxena et al. 2009; Weber et al. 2010).

Different lignocellulose pretreatments and/or enzymatic hydrolysis processes result in hydrolysates that contain pentoses (*e.g.* xylose) and hexoses (*e.g.* glucose) as the major fermentation substrates (Ahring et al. 1996; Chundawat et al. 2011; Saxena et al. 2009; Sun and Cheng 2002). Pretreatment also generates fermentation inhibitors, including furan derivatives such as furfural and hydroxymethyl furfural (HMF) from carbohydrate degradation, phenolic compounds from lignin, and aliphatic acids (acetic acid, formic acid and levulinic acid) (Saha 2003). The presence of fermentation inhibitors as well as the low substrate utilization for xylose-rich hydrolysates (Li et al. 2010; Lo et al. 2008; Long et al. 2010; Maintinguer et al. 2011) limit the direct and economical utilization of complex substrates such as biomass hydrolysates. A focused evaluation of the effects of different inhibitors on mixed culture hydrogen production showed furans and lignins having the greatest effect on maximum cumulative hydrogen production, while phenol caused the longest lag (Quéméneur et al. 2012). Substrate utilization and hydrogen production also decrease with increasing ethanol, acetic acid, propionic acid and butyric acids in mixed cultures (Wang et al. 2008b). However, *Clostridium beijerinckii* showed

more resistant to these inhibitors compared to other clostridial and non-clostridial bacteria (Quéméneur et al. 2012) and is thus a promising microorganism for hydrogen production from lignocellulosic hydrolysates. For example, for acetone, butanol and ethanol (ABE) production, *C. beijerinckii* BA101 was not inhibited by HMF and furfural at 3g/L (Ezeji et al. 2007).

Several approaches have been applied to improve biohydrogen production from lignocellulosic hydrolysates, including mixed cultures (Kongjan et al. 2009; Li and Fang 2007; Temudo et al. 2009; Valdez-Vazquez and Poggi-Varaldo 2009; Venkata Mohan 2009), genetically modified microorganisms (Jones 2008; Li et al. 2010; Maeda et al. 2008), defined co-cultures (Chang et al. 2008; Fang et al. 2006; Hsiao et al. 2009; Liu et al. 2008; Maintinguer et al. 2011; Wang et al. 2008a) and extracellular electron shuttle (EES) addition (Ye et al. 2012a; Ye et al. 2012b; Ye et al. 2011). Mixed cultures are beneficial for fermenting complex feedstocks due to i) reduced concerns about contamination resulting in simpler operational conditions, ii) more metabolic pathways allowing conversion of diverse substrates, and iii) better process stability for biowaste (Bartacek et al. 2007; Sinha and Pandey 2011). However, the optimal operational parameters vary, since the microbial communities change with different inocula and feedstock compositions. For example, contradictory optimal pH values (Fang and Liu 2002; Kim et al. 2004; Lee et al. 2002; Lin et al. 2008; Mu et al. 2006b; Wu and Lin 2004; Zhang et al. 2007) and hydraulic retention times (Sinha and Pandey 2011; Wang and Wan 2009; Wu et al. 2007; Wu et al. 2008a) are reported. In contrast, while working with pure cultures introduces operational complications, genetically modified microbes can achieve high hydrogen yield for a specific pure substrate (Chittibabu et al. 2006; Li et al. 2010; Liu et al. 2006; Oh et al. 2009). For example, with the overexpression of *hydA* in *Clostridium paraputrificum*, a hydrogen yield of 2.4 mol H_2/mol GlcNAc was achieved, a 70% increase over the wild type strain (Morimoto et al.

2005). With the aid of computational modeling and systems biology, optimal flux solutions for hydrogen production by genetically modified strains can be predicted before experimental validation (Becker et al. 2007; Jones 2008). Some researchers have also used co-culture fermentation to enhance biohydrogen production, taking advantage of different syntrophic or symbiotic interactions among species (Chang et al. 2008; Fang et al. 2006; Hsiao et al. 2009; Liu et al. 2008; Maintinguer et al. 2011; Wang et al. 2008a). In nature, syntrophic interactions between different groups of organisms are the norm rather than the exception.

The approach used in this work combines the addition of EES with a co-culture strategy for regenerating the reduced electron shuttle. Addition of the model EES anthrahydroquinone-2, 6-disulfonate (AH_2QDS) is known to increase hydrogen production for different pure substrates (glucose, xylose, cellobiose) in both *C. beijerinckii* pure culture (Ye et al. 2012a; Ye et al. 2012b; Ye et al. 2011) and a co-culture of *C. beijerinckii* and *Geobacter metallireducens* (Zhang et al. 2012b). The increased hydrogen production results from increased xylose utilization and reduced product (*e.g.* acetate) inhibition in co-culture. Co-culturing with *G. metallireducens* allows in-situ biological regeneration of the reduced EES and decreases accumulation of acetate (Zhang et al. 2012b). Although to date only AH_2QDS has been tested in this co-culture fermentation, *G. metallireducens* has been reported to use a range of EES, including juglone (Wolf et al. 2009b), lawsone (Lovley et al. 1998; Wolf et al. 2009b), fulvic acids (Fulton et al. 2004; Wolf et al. 2009b) and humic acids (Lovley et al. 1996; Wolf et al. 2009b), for bioremediation applications.

While previous studies with defined substrates and model EES have demonstrated the feasibility of the co-culture with EES approach, to apply this approach, it will be critical to effectively use complex substrates such as lignocellulosic hydrolysates and to identify less expensive EES. In this paper, we evaluate hydrogen production, substrate utilization, and acetate

accumulation from the co-culture with EES system using lignocellulosic hydrolysates with different glucose: xylose ratios and alternative EES.

4.3 Materials and Methods

4.3.1 Culture Maintenance

Clostridium beijerinckii NCIMB 8052 (TetraVitae Bioscience, Inc) was maintained in modified P2 medium (Baer et al. 1987) with 10mM glucose at 37°C and transferred every week to limit solvent production (Zhang et al. 2012b). *Geobacter metallireducens* GS-15 (lab collection) was maintained in freshwater medium (Lovley et al. 1993) at 30°C with 5mM anthraquinone-2, 6-disulfonate (AQDS) and 20 mM acetate. All media were sonicated and flushed with N_2 and then autoclaved at 121°C for 20 minutes.

4.3.2 Chemicals

AQDS was purchased from AKSCI (Union City, CA, USA). Indigo dye, juglone and humic acid were purchased from Acros Organics (New Jersey, USA). Fulvic acid was from Waterstone Technology (Carmel, IN, USA). Chemically reduced AH_2QDS was generated by sparging a solution of 5 mM AQDS in 30 mM bicarbonate buffer with H_2:CO_2 (80:20, vol/vol) for at least 1 h in the presence of 100 g/L palladium-coated aluminum catalyst as previously described (Ye et al. 2011). The AH_2QDS stock solution was then sparged with hydrogen-free N_2 and filtered twice through a 0.2 μm filter (PALL Acrodisc® syringe filter) into a sterile, anaerobic serum bottle. No hydrogen remained in the stock solution.

4.3.3 Dilute acid pretreatment

The lignocellulosic hydrolysates were derived from *Miscanthus x giganteus* biomass by a

hydrothermal pretreatment process with dilute acids in a 1.8 L pressure reactor (Guo et al. 2012) under different temperatures, pHs, and retention times (Table 4.1). The pH was adjusted by overliming with $Ca(OH)_2$ and H_2SO_4 up to 10 first and down to 5.5 subsequently (Guo et al. 2012). After filtering through a 0.2 μm filter, hydrolysate stocks were degassed and autoclaved as described for media above.

4.3.4 Batch experiments

Triplicate batch tests were performed with 10 mL of liquid in 26 mL anaerobic tubes. The composition was 10% hydrolysates, 10% *G. metallireducens* inoculum (in fresh water medium with 5mM AQDS) and 3% *C. beijerinckii* inoculum (in modified P2 medium) in modified fresh water medium. The tubes were incubated at 30°C in the dark and sampled every 4 h (Zhang et al. 2012b). H_2 was quantified from headspace samples, and the liquid samples were filtered through 0.2 μm syringe filters (PALL Acrodisc®) and stored at 4°C for future analysis.

Four fermentation conditions were carried out in experiment 1: co-culture, pure culture *C. beijerinckii* with chemically reduced AH_2QDS (0.5mM), *C. beijerinckii* without AH_2QDS, and an abiotic control. The initial total carbohydrate concentration was 2g/L.

Different pretreatment conditions resulted in different glucose and xylose abundance. To investigate the effects of the glucose: xylose ratio, in experiment 2 hydrolysates with glucose: xylose ratios equal to 1: 0.2, 1:1, and 1:10 were used as substrates to evaluate the hydrogen production and substrate utilization in both co-culture and *C. beijerinckii* pure culture. The initial total carbohydrate concentration was 3g/L.

To investigate the effectiveness of different EES, *G. metallireducens* was grown in freshwater medium with either 6 mM indigo dye, 1mM juglone, 1mM lawsone, 0.5g/L fulvic acids, or 0.25g/L humic acids as the electron acceptor, producing *G. metallireducens* inocula with

different biologically reduced EES. These inocula were used in co-culture batch tests with 2 g/L xylose as substrate.

4.3.5 Analytical Techniques

Analytical methods were as previously described (Guo et al. 2012; Zhang et al. 2012b). In brief, headspace hydrogen gas was measured by GC (Shimadzu GC-14A) equipped with a thermo conductive detector (TCD, SRI instrument Model 110). Filtered aqueous samples were analyzed by HPLC (Dionex Summit) with a Transgenomic® Organic Acid column for organic acids, GC-FID (Shimadzu-GC2014) with DB-FFAP capillary column for acetone, butanol and ethanol and HPLC (Shimadzu) with a refractive index detector (Waters, Milford, MA, USA) for xylose and glucose as described previously (Zhang et al. 2012b). The hydrolysates were analyzed for acetic acid, furfural and HMF by HPLC (Shimadzu) (Guo et al. 2012), and total phenols colorimetrically by the Folin-Ciocalteu method (Scalbert et al. 1989).

4.3.6 Kinetic modeling for hydrogen production, product formation and substrate utilization

The cumulative hydrogen production and substrate utilization were simulated by a modified Gompertz equation (Lay et al. 1998), resulting in calculated values for maximum hydrogen production $P_{max,i}$, hydrogen production rate or substrate utilization rate R_i, and lag phase λ_i, where i represents hydrogen or substrate.

$$P_i = P_{\max,i} \times \exp\{-\exp[\frac{\exp(1) \times R_i}{P_{\max,i}}(\lambda_i - t) + 1]\}$$

(3.1)$P_{hydrogen}$ and $P_{max,hydrogen}$ are in μmol; $P_{(max),\ glucose}$ and $P_{(max),\ xylose}$ are in mM; $R_{hydrogen}$ is in μmol/h; $R_{glucose}$ and R_{xylose} are in mM/h; λ_i is in hour for all the products.

4.3.7 Statistical analysis

Residual plots (Q'Q plots, correlogram) were applied to evaluate the assumption of normal distributed independent errors and therefore that the estimated parameter uncertainty is reliable. Based on the estimated parameters and their standard deviations, corresponding p-values were calculated to test if the parameters differ statistically significant for the different conditions.

4.4 Results

4.4.1 Lignocellulosic biomass after pretreatment as substrate

4.4.1.1 H_2 production and substrate utilization

Both *C. beijerinckii* plus AH_2QDS and co-culture with AH_2QDS improved hydrogen production (Table 4.2 experiment 1, Figure 4.1) when using the lignocellulosic hydrolysates derived from *Miscanthus x giganteus* biomass by dilute acid pretreatment as the substrate. Compared to *C. beijerinckii* alone, co-culture fermentation achieved up to a 25% increase in the maximum cumulative hydrogen production and a 41% increase in the specific hydrogen production rate, based on kinetic modeling, as well as a 44% increase in the specific xylose utilization extent according to xylose measurement (p-values < 0.1), while shortening the lag phase from 20 h to 18 h.

To specifically test the relationship between the glucose:xylose ratio in the hydrolysates and hydrogen production, lignocellulosic hydrolysates with different glucose:xylose ratios (1:0.2, 1:1, 1:10) were compared in batch tests. For all three hydrolysates, co-culture with AH_2QDS improved hydrogen production as compared to the pure culture of *C. beijerinckii* (Table 4.2 experiment 2, Figure 4.2). Based on the modeling results, the co-culture fermentation shortened the lag phase, increased the specific hydrogen production rates for all three hydrolysates and

increased substrate utilization rates for both glucose and xylose, unless the initial substrate concentration was low, less than 0.4 g/L xylose (S2 samples) or less than 0.3g/L glucose (S4 samples). According to the experimental results for hydrogen and substrate measurement, the improvement in maximum cumulative hydrogen from pure culture to co-culture system was larger when fermenting samples with lower glucose:xylose ratios (22% increase for S3 and 11% increase for S4), while maximum cumulative hydrogen production was similar between pure culture and co-culture conditions for the samples with higher glucose:xylose ratio (S2). The hydrogen production profiles matched the substrate utilization efficiency results. Hydrolysates with lowest glucose:xylose ratio (1:10 in S4) achieved the highest improvement (18%) in xylose utilization. Considering these results, using a co-culture system increased the extent of xylose utilization. There was no significant difference for the extent of glucose utilization for all three conditions; almost complete glucose consumption was achieved regardless of the presence of AH_2QDS or *G. metallireducens*.

4.4.1.2 Acetate production and utilization

Acetate did not accumulate in the co-culture system during fermentation of lignocellulosic hydrolysates (Figure 4.3). Initial acetate concentrations resulted from the pretreatment products (6-7 mM) and the *G. metallireducens* inoculum (about 0.6 mM acetate). In the *C. beijerinckii* pure culture system and *C. beijerinckii* with AH_2QDS system, acetate increased in the log phase and remained relatively stable thereafter, paralleling the fermentative hydrogen production profile. In the co-culture system, a significant decrease of acetate concentration from 12 mM to 4 mM at the end of the fermentation was observed, resulting from acetate consumption by *G. metallireducens*.

4.4.2 Application of alternative extracellular electron shuttles

The EES indigo dye, juglone, lawsone, fulvic acids and humic acids, were investigated in co-culture fermentations with xylose as the substrate. All EES increased biohydrogen production more with the co-culture than the *C. beijerinckii* pure culture, with 90% confidence interval (Table 4.3). With these extracellular electron shuttles, the co-culture system achieved a 61-98% increase in the maximum cumulative hydrogen production, a 157-368% increase in the specific hydrogen production rate, a 14-45% increase of hydrogen molar yield and a 63-72% increase in the substrate utilization extent, while having a shorter lag phase. Therefore, all the tested EES improve biohydrogen production in co-cultures of *C. beijerinckii* and *G. metallireducens.*

4.5 Discussion

These data demonstrate that hydrogen production from fermentation of lignocellulosic hydrolysates increases through the use of a co-culture system of *C. beijerinckii* and *G. metallireducens* amended with AH_2QDS or alternate electron shuttles. The improvement was greater for the fermentation of xylose-rich hydrolysates. Several EES were substituted for AH_2QDS in the co-culture system with comparable results. These results advance previous work on the co-culture system, which exclusively used simple substrates and model EES (Zhang et al. 2012b), and demonstrate its utility with more realistic substrates and EES.

The co-culture system resulted in the greatest improvements for xylose-rich hydrolysates because glucose consumption was essentially complete even for the pure culture conditions. In other words, there was no room for improvements in glucose utilization under the conditions tested. Hydrogen production increased due to increased xylose utilization and perhaps also diminished acetate inhibition. The importance of the glucose:xylose ratio in fermentations has

been noted previously, but the trend is not consistent, with for example a pure culture ethanol fermentation showing a preference for glucose over xylose (Zhao et al. 2008) (Prakasham et al. 2009; Prakasham et al. 2010), while other reports using a consortia showed more hydrogen production from xylose. Our results illustrate the advantage of the co-culture system for fermenting xylose-rich hydrolysates, supporting previous studies using simple substrates (Ye et al. ; Zhang et al. 2012b).

Compounds such as furans and phenols are common in lignocellulosic hydrolysates and can inhibit fermentation processes (Cao et al. 2010; Quéméneur et al. 2012). Furans were present at concentrations of 0.063-0.980 g/L HMF and 1.54-6.96 g/L furfural in the hydrolysates used here (Table 4.1). Although phenols were not quantified in these samples, they are expected to be present at concentrations of approximately 2.32-4.51 g/L in gallic acid, based on their abundance in other hydrolysates derived from the same type of biomass and pretreatment process (data not published). Although the inhibitory concentrations vary for different microbes, phenolic compounds at concentrations of 1 g/L inhibited growth and H_2 or ethanol production in yeast and bacteria (Delgenes et al. 1996; Ezeji et al. 2007; Quéméneur et al. 2012). However, in the current work H_2 was produced despite the presence of fermentation inhibitors in both pure culture and co-culture conditions, suggesting that this system may be relatively insensitive to common inhibitors.

The co-culture system also consumes acetate by *G. metallireducens* significantly, which could increase hydrogen production by reducing product inhibition, especially for a continuous production mode. Acetate accumulation affects hydrogen production in two ways. First, the accumulation of volatile organic acids (*e.g.* acetic acid and butyric acid) can trigger a shift from the acidogenic phase to the solventogenic phase and lower hydrogen production in *Clostridium*

fermentation (Gottschal and Morris 1981; Terracciano and Kashket 1986; Van Ginkel and Logan 2005). Addition of acetate has even been used as a strategy to induce early solvent production (Gottschal and Morris 1981) or enhance solvent production and prevent degeneration (Chen and Blaschek 1999) in *Clostridium.* However, the strain used in this work was defective in solvent production, so this effect is not relevant here. Second, at higher concentration, acetate can significantly inhibit growth (for example at concentrations above 100 mM in *Clostridium* (Zhang et al. 2012a)) and above 50 mM for *Ethanoligenensi* (Tang et al. 2012), although some clostridia tolerate to up to 60 mM acetate during solvent production (Chen and Blaschek 1999). Acetate concentrations in our batch tests were in the range of 8 – 12 mM, so inhibition may not have been occurring under our experimental conditions.

The significant increase of maximum hydrogen production, hydrogen production rate, hydrogen molar yield and substrate utilization extent (Table 4.3, Figure 4.4) with all tested EES illustrated that a variety of EES can conduct the electron shuttling between *C. beijerinckii* and *G. metallireducens*, resulting in increased hydrogen production. The EES requirements in the co-culture system are not specific, since both quinone type (juglone, lawsone, fulvic acids and humic acids) and non-quinone type (indigo dye) EES significantly improved hydrogen production in the co-culture system. These results demonstrate the utility of the co-culture system with EES that are less expensive than the model electron shuttle AH_2QDS. The observed lack of specificity suggests that raw humic acids may also serve as EES in the co-culture system.

4.6 Conclusion

We evaluated the feasibility of using a co-culture system of *G. metallireducens* and *C. beijerinckii* with EES for enhanced biohydrogen production from complex biomass-derived substrates. This co-culture system improved xylose utilization and reduced acetate accumulation

when fermenting xylose-rich lignocellulosic hydrolysates from diluted acid pretreatment of *Miscanthus x giganteus*. The presence of known fermentation inhibitors such as furans did not prevent hydrogen production. A wide range of EES were also functional in the co-culture system. In combination with existing industrial applications using lignocellulosic hydrolysates in ethanol production and EES in bioremediation, which demonstrate the availability of those materials for large scale utilization, these results support the feasibility of large-scale biohydrogen production using this co-culture system.

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Table 4.1 Pretreatment conditions and characteristics for different hydrolysate samples [a]

Hydrolysates	Glucose:xylose ratio	Catalysts	Conditions	Inhibitors (g/L) [b]
S1	~1:5	1.46 wt% H_2SO_4	150°C, 6min, IP 97 psi	0.127 HMF, 4.64 furfural, 3.72 acetic acid
S2	~1:0.2	1.0 wt% H_2SO_4	180°C, 20min, IP 94 psi	0.980 HMF, 6.96 furfural, 7.52 acetic acid
S3	~1:1	1.0 wt% H_2SO_4	160°C, 35min, IP 94 psi	0.331 HMF, 4.45 furfural, 5.30 acetic acid
S4	~1:10	0.365 wt% H_2SO_4 + 4 ml/L TFA	150°C, 6min, IP 3 psi	0.063 HMF, 1.54 furfural, 5.96 acetic acid

[a]The feedstocks for the hydrolysates were 120g *Miscanthus*, 20% Dry matter content and all the pretreatment experiments are carried out in the pressure reactor (Model 4543). TFA stands for trifluoroacetic acid; IP stands for initial pressure when the reaction started

[b]Phenolics were not measured for these 4 samples, but were likely to be present based on analysis of other samples derived from the same type of biomass and pretreatment methods (hydrothermal treatment with diluted acids). The detected total phenols for the other samples were in the range of 2.32-4.51 g/L in gallic acid (Chen and Blaschek 1999).

Table 4.2 Hydrogen production and substrate utilization using hydrolysates from dilute acid pretreatment with different glucose: xylose ratios as substrate

Condition	Total sugar (g/L)	Glucose:xylose ratio	Lag phase λ_i (h) [a]	Maximum H_2 production $P_{max,hydrogen}$ (μmole) [a]	Specific H_2 production rate $R_{hydrogen}/V$ (mmol/L/h) [a]	Xylose utilization rate $R_{substrate}$ (g/L/h) [a]	Glucose utilization rate $R_{substrate}$ (g/L/h) [a]	Xylose utilization% [b]	Glucose utilization% [b]
Experiment 1	3								
S1, *C. beijerinckii* alone		~1:5	20.0± 0.5	230± 5	1.72±0.15	n.d.	n.d.	61.4±4.7%	100±0%
S1, *C. beijerinckii* + AH_2QDS		~1:5	18.4± 0.5	245± 5	2.24±0.23	n.d.	n.d.	70.5±1.2%	99.0±1.8%
S1, Co-culture		~1:5	18.2± 0.8	287± 9	2.43±0.34	n.d	n.d	88.5±1.7%	97.5±0.6%
Experiment 2	2								
S2, *C. beijerinckii* alone		~1:0.2	28.6±0.5	182±3	1.12±0.09	0.073±0.001	0.222±0.012	100±0%	99.5±0.0%
S2, Co-culture		~1:0.2	24.1±0.5	179±3	1.45±0.13	0.079±0.000	0.240±0.052	100±0%	99.8±0.0%
S3, *C. beijerinckii* alone		~1:1	24.9±0.7	151±4	1.15±0.16	0.110±0.009	0.147±0.016	96.1±0.3%	98.6±0.2%
S3, Co-culture		~1:1	21.6±0.4	184±3	1.67±0.14	0.151±0.011	0.182±0.015	97.7±0.1%	98.5±0.1%
S4, *C. beijerinckii* alone		~1:10	21.4±0.5	159±4	0.73±0.05	0.094±0.002	0.033±0.005	66.5±0.7%	98.1±0.0%
S4, Co-culture		~1:10	16.8±0.6	176±3	0.80±0.06	0.117±0.001	0.035±0.005	78.7±0.5%	98.3±0.0%

The "±" in the table stands for the standard error (residuals) for the estimation of the parameters. According to the statistical analysis, the model parameters are valid since (i) their residuals are normal distributed; (ii) the variance is more or less constant; and (iii) the residuals are independent.

[a]: kinetic parameters from the modified Gompertz model

[b]: parameters calculated directly from the experimental results. Substrate (xylose or glucose) utilization% refers to the moles of substrate consumed versus the initial moles of substrate during a 60 hours batch test.

n.d.: not determined

Table 4.3 Kinetic parameters for hydrogen production with different EES

Condition	Type of EES	Lag phase, λ_i (h) [a]	Maximum H_2 production, $P_{max,hydrogen}$ (μmole) [a]	Specific H_2 production rate, $R_{hydrogen}/V$ (mmol/L/h) [a]	H_2 molar yield (mol H_2/mol xylose) [b]	Xylose utilization% [b]
C.beijerinckii alone		23.8±1.9	158.9±16.1	0.44±0.06	1.88±0.13	58.2±0.3%
Co-culture, indigo dye	Non-quinone	21.7±0.7	268.8±6.7	1.61±0.16	2.15±0.03	100±0%
Co-culture, juglone	Quinone	21.6±2.1	289.2±24.6	1.18±0.23	2.41±0.14	99.9±0.02%
Co-culture, lawsone	Quinone	20.4±1.2	300.2±14.2	1.13±0.12	2.51±0.09	99.9±0.1%
Co-culture, fulvic acids	Quinone	20.5±01.2	315.1±11.7	2.06±0.35	2.72±0.13	100±0%
Co-culture, humic acids	Quinone	18.2±1.1	255.7±38.0	1.51±0.22	2.38±0.11	94.8±2.0%

The "±" in the table stands for the standard error (residuals) for the estimation of the parameters. According to the statistical analysis, the model parameters are valid since (i) their residuals are normal distributed; (ii) the variance is more or less constant; and (iii) the residuals are independent

[a]: kinetic parameters from the modified Gompertz model

[b]: parameters calculated directly from the experimental results. Substrate (xylose or glucose) utilization% refers to the moles of substrate consumed versus the initial moles of substrate during a 60 hours batch test

n.d.: not determined

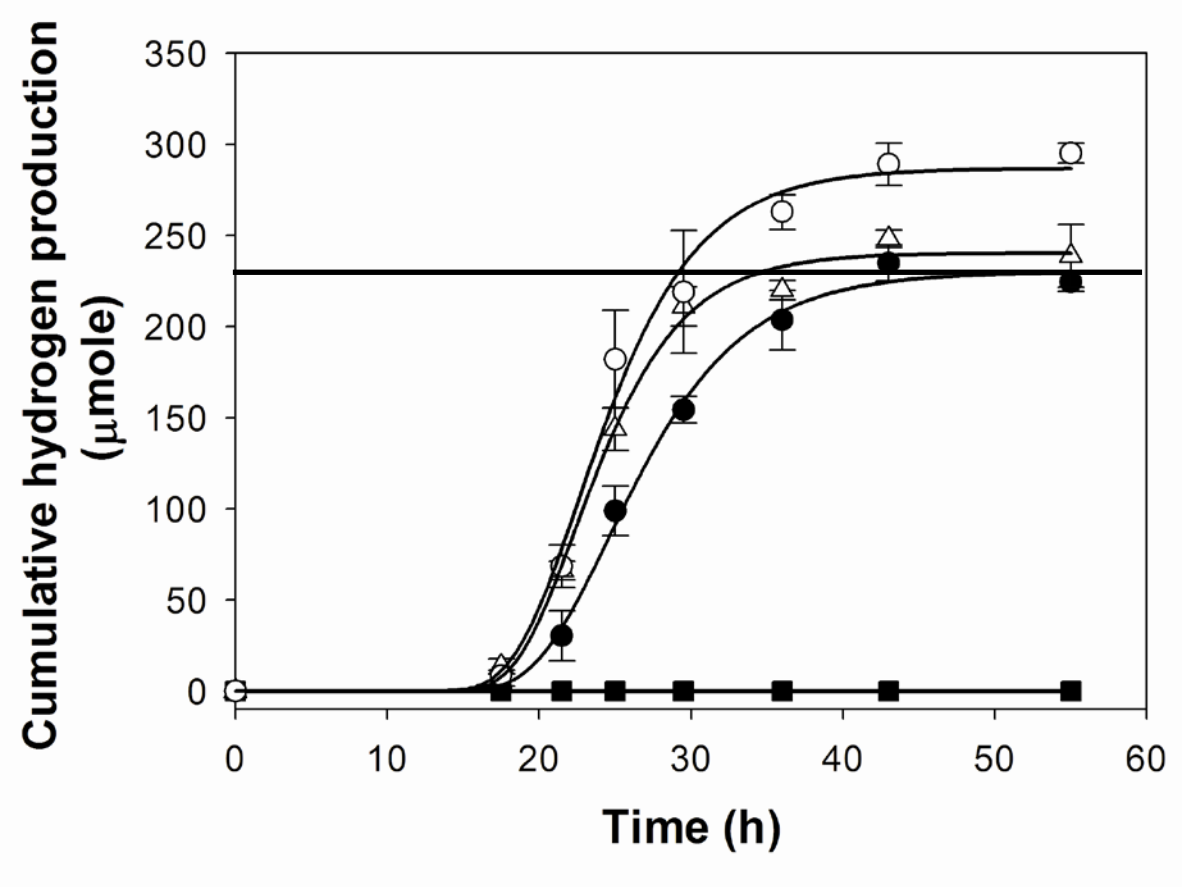

Figure 4.1 Experimental data and kinetic model fits of cumulative hydrogen production versus time using hydrolysate from dilute acid pretreatment (glucose: xylose=1: 5) as substrate. Data points are the means of triplicate cultures. Error bars refer to one standard deviation. Lines are the fitted curves of the Modified Gompertz equation (Eq 4.1). The horizontal line marks the maximum hydrogen production by *C. beijerinckii* without AH_2QDS. ○: *C. beijerinckii* and *G. metallireducens* with AH_2QDS; △: *C. beijerinckii* + AH_2QDS; ◇: *C. beijerinckii* alone; ■: Abiotic control.

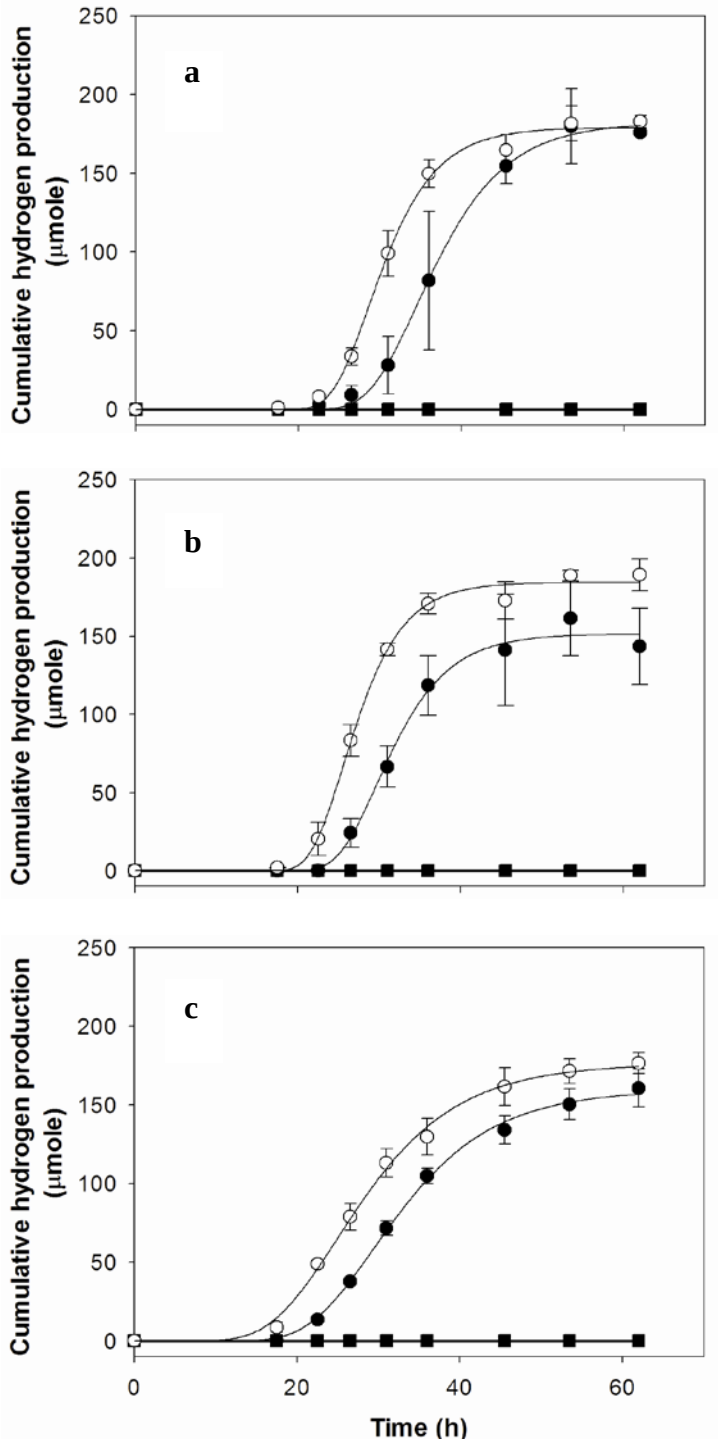

Figure 4.2 Experimental results and kinetic model fits of cumulative hydrogen production versus time using hydrolysate with different glucose:xylose ratios in the substrate (a: 1:0.2, b: 1:1, c: 1:10). Data points are the means of triplicate cultures. Error bars refer to one standard deviation. Lines are the fitted curves of the Modified Gompertz equation (Eq 3.1). ○**:** *C. beijerinckii* and *G. metallireducens* with AH_2QDS; ●: *C. beijerinckii* alone; ■: Abiotic control

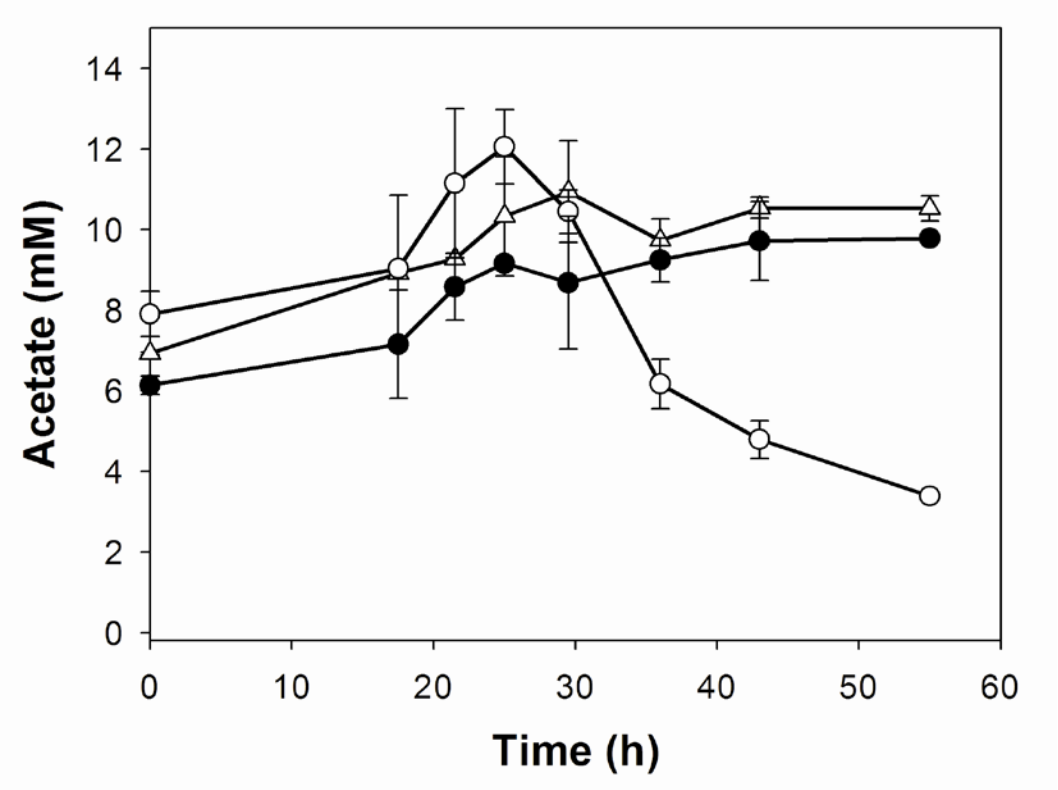

Figure 4.3 Profile of acetate versus time. Data points are the means of triplicate sampling values. Bars refer to one standard deviation. ○: *C. beijerinckii* and *G. metallireducens* with AH_2QDS; ∆: *C. beijerinckii* + AH_2QDS; ■: *C. beijerinckii* alone.

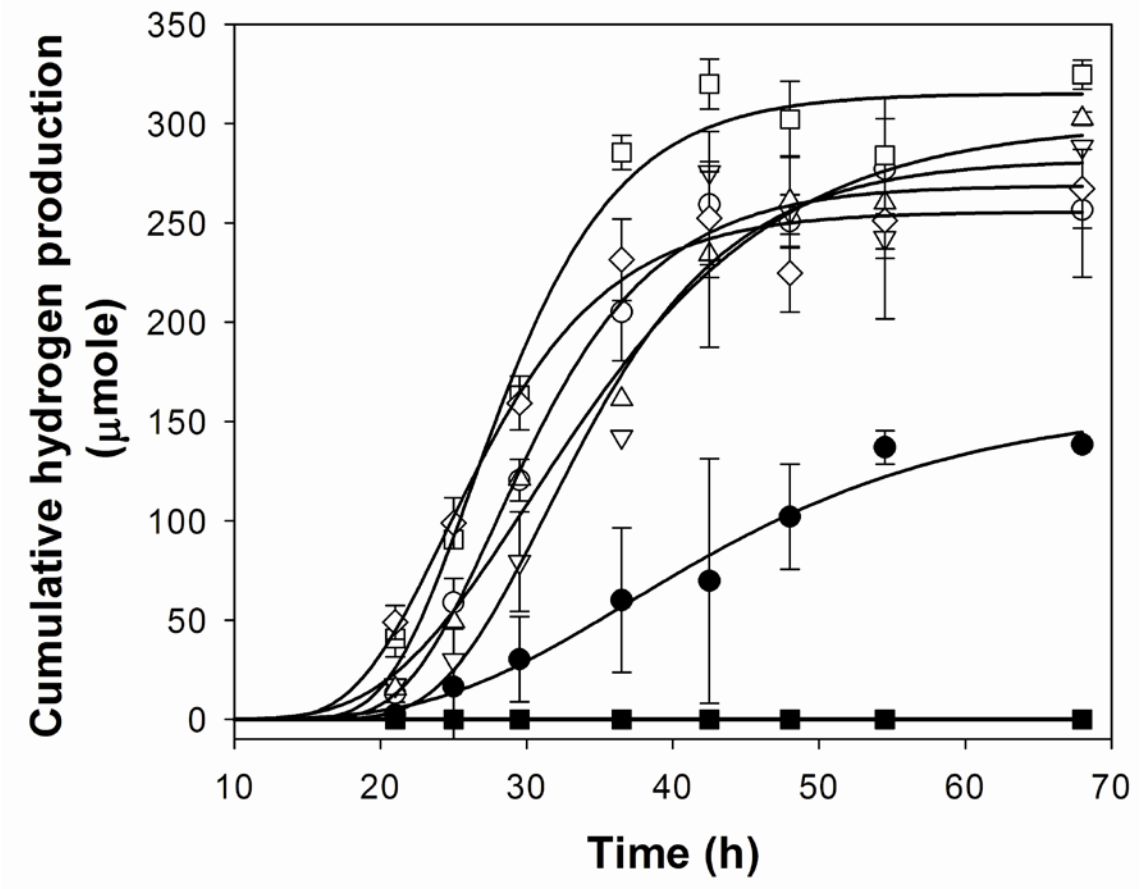

Figure 4.4 Experimental data and kinetic model fits of cumulative hydrogen production versus time using different extracellular electron shuttles. Data points are the means of triplicate cultures. Error bars refer to one standard deviation. Lines are the fitted curves of the Modified Gompertz equation (Eq 3.1). Co-culture stands for *C. beijerinckii* and *G. metallireducens*. ○**:** Co-culture + indigo dye; ▽: Co-culture + juglone; △: Co-culture + lawsone; ◇: Co-culture + humic acids; □: Co-culture + fulvic acids; ●: *C. beijerinckii* alone; ■: Abiotic control.

CHAPTER 5 CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The objective for this work was to develop an applicable biohydrogen production strategy. The relatively low H_2 production rate, H_2 molar yield, and substrate (especially xylose) utilization are the major limitations for industrial biohydrogen production and there is a knowledge gap of the syntrophy with electron shuttles for fermentative biohydrogen production. We proposed a novel co-culture system (*C. beijerinckii* and *G. metallireducens*) in the presence of the reduced extracellular electron shuttle (EES) to increase fermentative hydrogen production from xylose-rich substrates. The interaction between these two cultures and the influence on hydrogen production was investigated using a model substrate (xylose) and an extracellular electron shuttle (AH_2QDS). In addition, the feasibility of economical application of this co-culture with EES system was evaluated with practical substrates (lignocellulosic hydrolysates) and different EES.

The metabolic crosstalk between *C. beijerinckii* and *G. metallireducens* was demonstrated in a simplified co-culture system with the model EES AH_2QDS and using xylose as substrate. The major evidence included the improvement of *Clostridium* hydrogen production with the presence of AH_2QDS, the growth of *G. metallireducens* and utilization of *Clostridium* fermentation product acetate by *G. metallireducens* for AH_2QDS regeneration. In addition, the co-culture system achieved increases of 52.3% for the maximum cumulative hydrogen production, 38.4% for the specific hydrogen production rate, 34.8% for the hydrogen molar yield, 15.4% for the substrate utilization rate, and 39.0% for the substrate utilization extent in co-culture fermentation compared to a pure culture of *C. beijerinckii* without AH_2QDS.

These results demonstrate that the co-culture with AH_2QDS system can improve hydrogen

production from xylose. They are also consistent with previous findings that AH_2QDS addition improved hydrogen production by affecting *Clostridium* fermentation pathway and demonstrate in addition that i) biologically reduced AH_2QDS functions similarly to chemically reduced AH_2QDS, and ii) co-culture with *G. metallireducens* can further improve hydrogen production with the interaction between *C. beijerinckii* and *G. metallireducens*. Therefore, the co-culture with AH_2QDS system realized the regeneration of the reduced form electron shuttle AH_2QDS and overcame the obstacle of making and continuously regenerating AH_2QDS. This study of the conceptual model for co-culture with EES system suggested syntrophic co-culture fermentation in the presence EES is an attractive research direction for enhanced industrial hydrogen production.

To make the application co-culture with EES system more feasible, complex substrates and alternative electron shuttles were investigated. Lignocellulosic hydrolysates provide abundant substrates for fermentative hydrogen production, but are difficult to ferment. Co-culture with AH_2QDS showed great potential to overcome the obstacles of fermenting lignocellulosic hydrolysates by improving substrate utilization and diminishing product (acetate) inhibition. Up to a 24.7% increase in the maximum hydrogen production, a 41.4% increase in the hydrogen production rate, and a 44.0% increase in xylose utilization extent were achieved in co-culture compared to *C. beijerinckii* alone. The investigation of hydrolysates with different glucose:xylose ratios suggested such improvements were especially effective for xylose-rich hydrolysates (i.e., low glucose:xylose ratios). This improvement on fermentability of lignocellulosic hydrolysates supports the feasible application of co-culture with EES system to solve the difficulties of efficient utilization of lignocellulosic hydrolysates in industry.

The results of using different electron shuttles such as humic acid demonstrated the EES in

the co-culture are not required to be AH_2QDS and other types of EES (both quinone type and non-quinone type) played the same role as AH_2QDS in co-culture system to improve the hydrogen production significantly. For all conditions, co-culture with EES system achieved a 60.9-98.3% increase in the maximum cumulative hydrogen production, a 157-368% increase in the specific hydrogen production rate, 14-45% increase of hydrogen molar yield and 63-72% increase in the substrate utilization extent compared to pure culture without EES. The replacement of AH_2QDS by alternative EES, especially the ubiquitous and the much cheaper humic acids makes the co-culture with EES system more economical and flexible without relying on the amended chemicals.

Therefore, the improvement on the fermentability of xylose-rich hydrolysates and application of economical electron shuttles make the co-culture system with EES system a more practical strategy for industrial biohydrogen production.

5.2 Future work

This research established the co-culture with EES system for enhanced fermentative biohydrogen production. Two research directions are relevant for further evaluation.

5.2.1 Fundamental metabolism

We have found AH_2QDS improved *Clostridium* hydrogen production through a metabolic shift from the butyric acid pathway to the acetic acid pathway and an increase of hydrogen evolution rates from glyceraldehyde-3-phosphate, while AQDS increased *Clostridium* butanol production at metabolic level. However, more evidences in enzymatic or genetic level are needed to support these conclusion. In addition, although we believe AH_2QDS increases the biohydrogen production through a global influence on fermentation pathway, we cannot rule out the

possibility that it may partially act as direct electron donor to hydrogen. Hence, more investigation is required to further clarify the role of reduced form electron shuttles (*e.g.* AH_2QDS) in the *Clostridium* fermentation pathway and the co-culture crosstalk. Better understanding this mechanism will also help to optimize the type and concentration of EES addition for further application.

Experiments to investigate the enzymatic activity (*e.g.* hydrogenase, NADH-fd oxidoreductase) and track the fate of proton by radioactive label may help to solve those questions. Another interesting direction is using system biology model to simulate the effects of AH_2QDS on hydrogen production and predict the optimal operation parameters. The challenges are as an electron shuttle, the net formation and consumption of AH_2QDS equals to zero, while the existing models require a non-zero production or reaction equation as a constrain for the simulation.

5.2.2 Practical application

To apply this strategy, process optimization is necessary for scaling up the co-culture with EES system in a fed-batch or continuous mode. Fermentation parameters such as concentration of substrate and EES, types of EES, dilution rate, and *C. beijerinckii*: *G. metallireducens* inoculum ratio as well as reactor configuration should be investigated to achieve optimal hydrogen molar yield, production rate and substrate utilization for the scale-up implementation. To make the system more cost effective, we need to decrease the overall amount of EES dosage. Therefore, strategies for EES immobilization or recycle should also be investigated.

Some bacteria such as *Shewanella* can produce soluble extracellular shuttles/flavins. If *Clostridium* can be co-cultured with such kinds of microbes, the cost and operation complication will decrease significantly and the possibility of continuous operation increases since no

additional EES is needed for the co-culture system.

The sensitivity and tolerance to different inhibitors in lignocellulosic hydrolysates for the co-culture with EES system need to be evaluated. We have found that the co-culture with EES system improve biohydrogen production in the presence of fermentation inhibitors (furfural, HMF, phenols and acetate) when using lignocellulosic hydrolysates as feedstocks. However, further investigation for the effects of specific inhibitors is necessary.

We can also explore suitable downstream hydrogen purification technologies for co-culture with EES system. The rapid removal of headspace hydrogen is necessary to avoid gas inhibition for scale-up reactor, which however will increase the cost of downstream separation by decreasing hydrogen content in the gas phase. Therefore, downstream separation processes from CO_2 and carrier gas are important for scale-up biohydrogen production.

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APPENDIX A BIOHYDROGEN PRODUCTION BY *C.* BEIJERINCKII WITH AH_2QDS ADDITION IN THE CHEMOSTAT

A.1 Reactor configuration and operation conditions

A.1.1 Reactor configuration

A 1L Sartorius* BIOSTAT* A plus fermentor (Sartorius North America, Edgewood, NY) was used for the continuous experiments and BioPAT* MFCS/DA was used for system control (temperature, pH, flow rate for gas and liquid) and data acquisition (Figure A1). The fermentor and tubings was autoclaved at 121°C for 20 minutes before the experiments. N_2 was sparging to the reactor to keep anaerobic condition. HCl (4N) and NaOH (4N) was used for pH control. All liquid and gas was filtered by 0.2 μm filter (PALL Acrodisc® syringe filter) before entering the reactor. The reactor was operated in batch mode till the end of lag phase by monitoring the culture growth, and then shifted to continuous mode. AH_2QDS was pulse dosed into reactor after the hydrogen production entered steady state. AQUASIM can be used to model the culture growth, substrate utilization and product production at given condition.

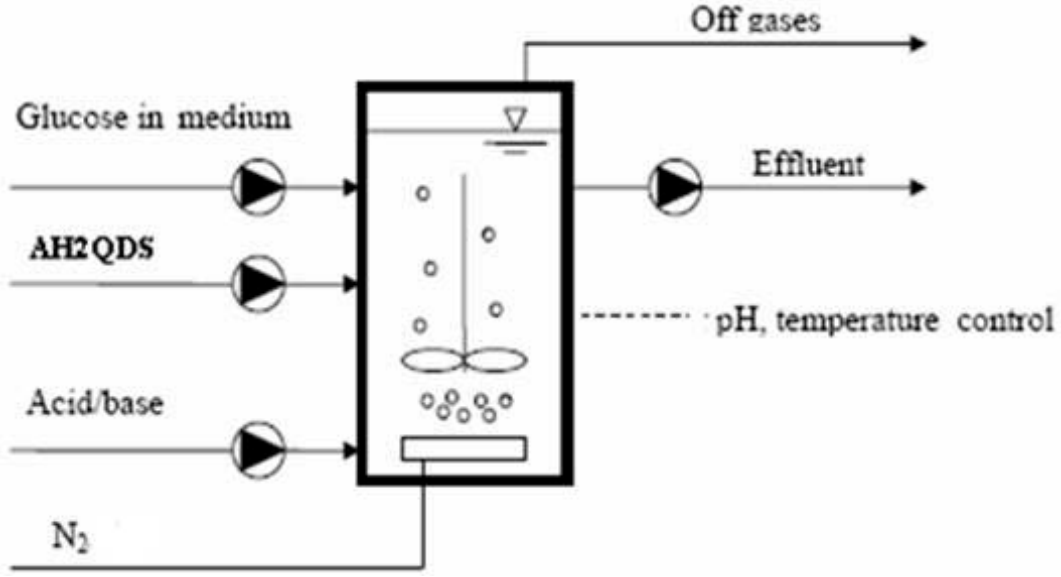


Figure A1 Configuration of chemostat for biohydrogen production by C. beijerinckii with AH_2QDS addition

A.1.2 Operation conditions

The operation condition for the chemostat was summarized in Table A1.

Table A1 Operation parameters for the chemostat for biohydrogen production by *C. beijerinckii* with AH_2QDS addition

Working volume	0.8 L
Culture	*C. beijerinckii* NCIMB 8052
Temperature	37 ºC
pH	7, 6.5, 6
Hydraulic retention time (HRT=1/dilution rate)	1.7 d, 1.5 d, 1 d
Flow rate of gas (N_2) sparging into the reactor	0.6 L/h, 0.4 L/h
Substrate concentration	modified P2 medium + 10 mM Glucose
Initial AH_2QDS concentration after pulse dosage	0.5 mM

A.2 Results

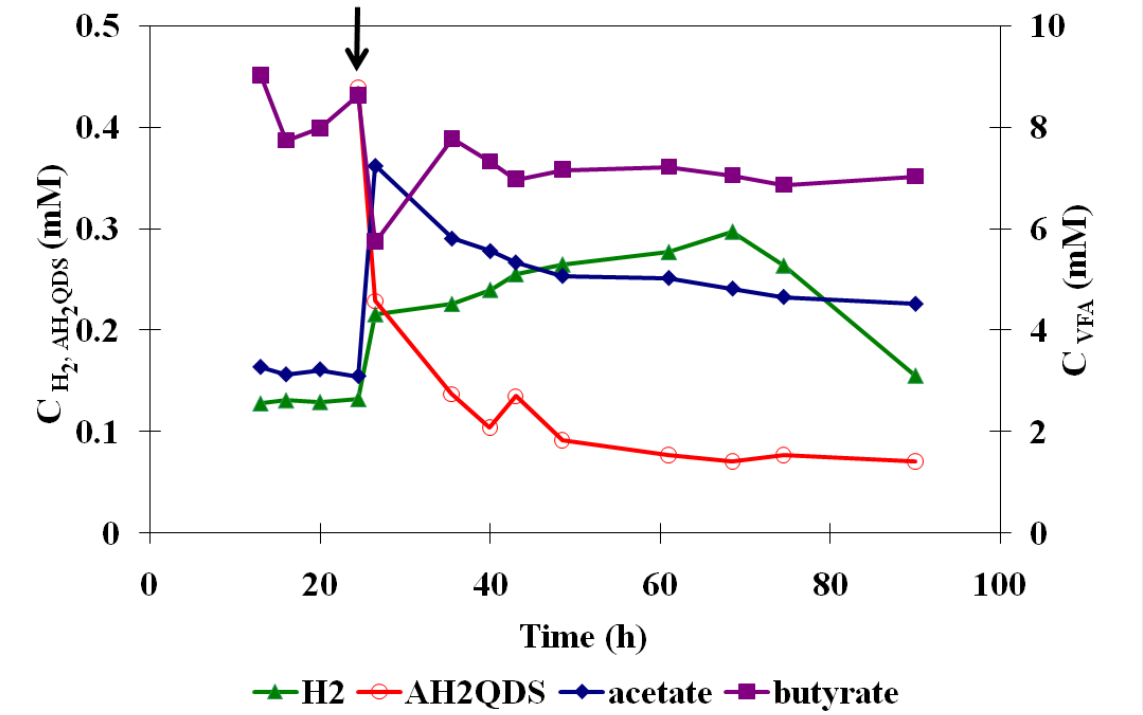


Figure A2 Influence of AH_2QDS (pulse dosage) on fermentation products in chemostat (T=37 ºC, pH=7, HRT=1.7 d, Q_{N2}=0.6 L/h)

APPENDIX B STATISTICAL ANALYSIS

Free software R (http://www.r-project.org/) was used for all the statistical analysis

B.1 Codes to prove the standard deviations for the nonlinear regression from the model are valid

The modified Gompertz equation is a nonlinear regression. The standard deviations for the estimated model parameters from the model are valid if

a) the residuals are normal distributed:

This is not so critical and tested visually with qqplot. All data points is close to the line in the qqplot if the data are normally distributed.

b) the variance is more or less constant

Plot residual error as function of predicted values and the variance is constant if residuals are randomly distributed

c) the residuals are independent

This is the most critical assumptions, and checked by ploting the autocorrelation functions (correlogram)). All the bars need to below the blue line.

B.1.1 Codes:

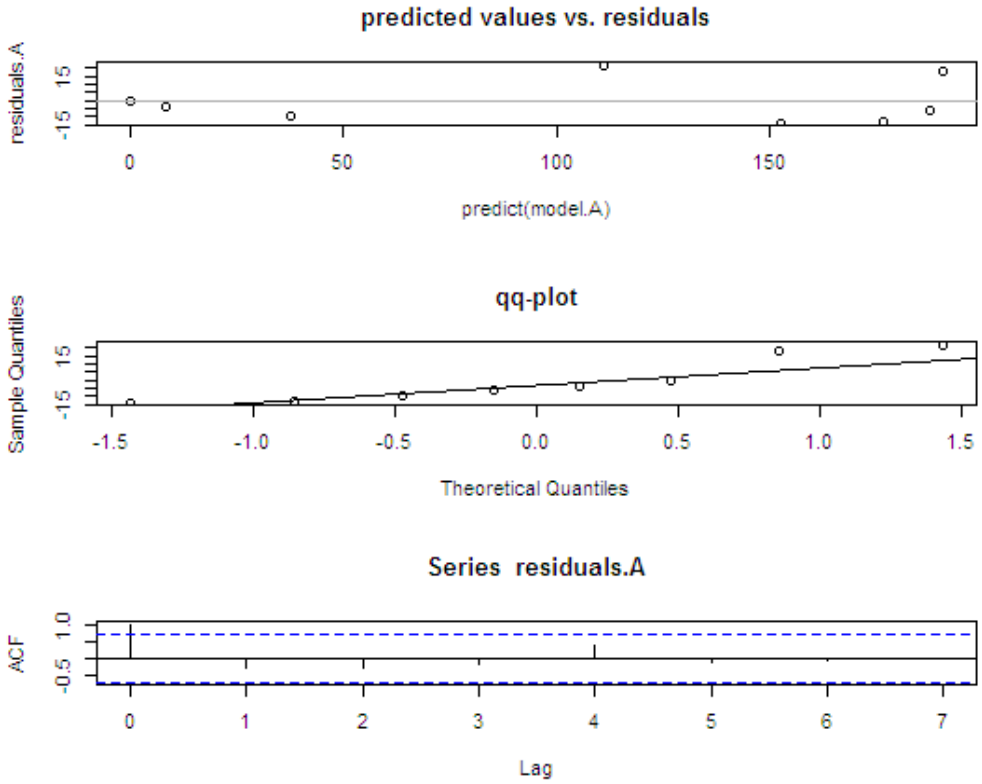
```
## ======================================================
## Fit a non linear model and check residuals
## ======================================================
## 1) read data
## the model results were saved in a CSV file and read to the R workspace
for analysis
myfile <- file.choose()
```

```
myfile
data <- read.table(myfile, header=TRUE, sep=";")
## ------------------------------------------------------
## have a look at the data
data
## ------------------------------------------------------
## plot some data
plot(data$t, data$P.a)
points(data$t, data$P.b, col='red')
## ======================================================
## 2) fit the models
##use nls() function to determine the nonlinear least-squares estimates
of the parameters of a nonlinear models (modified Gompertz equation). A,
B, C, D, E, F refers to different models for different conditions
## note, that you have to use "~" instead of "=" in the equation
model.A <- nls(P.a ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
data=data, start=c(P.max=200, R=10, lambda=10))
model.B <- nls(P.b ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
data=data, start=c(P.max=200, R=10, lambda=10))
model.C <- nls(P.c ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
data=data, start=c(P.max=200, R=10, lambda=10))
model.D <- nls(P.d ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
data=data, start=c(P.max=200, R=10, lambda=10))
model.E <- nls(P.e ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
data=data, start=c(P.max=200, R=10, lambda=10))
model.F <- nls(P.f ~ P.max * exp(-exp(exp(1)*R/P.max*(lambda-t)+1)),
```

```
data=data, start=c(P.max=200, R=10, lambda=10))
## ------------------------------------------------------
## shows you the paraemter, p-values, ect.
summary(model.A)
summary(model.B)
summary(model.C)
summary(model.D)
summary(model.E)
summary(model.F)
## ======================================================
## 3) look at the residuals
## Note, that with only 7 data points the judgment of the plot is very
difficult. However, strong patter would be visible.
## use result of A as an example
## get residuals for A
residuals.A <- resid(model.A)         #or you could do it by hand: data$P.a
- predict(model.A)
## ------------------------------------------------------
## --- plots for model A
par(mfrow=c(3,1))              # make three plots in one window
## ------------------------------------------------------
## i) predicted values vs residuals
plot(predict(model.A),   residuals.A,   main='predicted   values   vs.
residuals')
abline(h=0, col=gray(0.7))           # add a horizontal line at 0
```

```
## Here you want to see randomly distributed residuals along the x-axis.
## It's more or less ok here.

## -------------------------------------------------------
## ii) qqplot
qqnorm(residuals.A, main='qq-plot')
qqline(residuals.A)

## If the data are normally distributed, all points lie very close to the
line.
## As we have very few data, it is ok here

## -------------------------------------------------------
## iii) correlogram
acf(residuals.A)

## That look good here. We would have a problem if the bars go over the
blue lines
## (the first one doesn't count, it is always equals one.)

## =======================================================
## repeat for the residuals of models B, C, D, E, F (detailed codes were
not shown)
## =======================================================
```

B.1.2 Results for the R codes above:



B.2 Codes to determine whether significant differences existing among model results for different conditions

Since here are no strong correlation so that means for estimated parameters can be compared.

1 Compute the mean (mean.X and mean.Y) of the parameters and the standard deviation of the means for both groups.

2 Calculate the difference of means (Z) and the standard deviation of Z

3 Test if Z is significant different of 0, i.e. if the p.value is small enough

Codes:

```
## =======================================================
## Comparison of means for estimated parameters
## =======================================================
## max. hydrogen production
## --- parameters from first group (co-culture)
X <- c(198.2, 223.1, 212.8)         # estimated means (e.g. max. hydrogen
production)
X.sd <- c(10.9, 2.9, 2.7)               # estimated standard deviations
mean.X <- mean(X)                     # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## -------------------------------------------------------
## --- parameters from second group (pure culture+ AH2QDS)
Y <- c(174.3, 184.3)                    # estimated means
Y.sd <- c(4.2, 3.7)                     # estimated standard deviations
mean.Y <- mean(Y)                       # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))          # standard deviation of mean.Y
## -------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## -------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
## Ha: difference (Z) not = 0
```

```
p.value  <-  2*(1-pnorm(abs(Z)/sd.Z))          #probability  of  normal
distribution
p.value


## ========================================================
## lag phase
## -- parameters from first group (co-culture)
X <- c(10.39, 13.16, 13.43)             # estimated means
X.sd <- c(2.24, 0.53, 0.51)              # estimated standard deviations
mean.X <- mean(X)                      # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## --------------------------------------------------------
## ---- parameters from second group (pure culture+ AH2QDS)
Y <- c(20.23, 19.23)                    # estimated means
Y.sd <- c(0.69, 0.76)                    # estimated standard deviations
mean.Y <- mean(Y)                       # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))          # standard deviation of mean.Y
## --------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## --------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
## Ha: difference (Z) not = 0
p.value <- 2*(1-pnorm(abs(Z)/sd.Z))
```

```
p.value

## ========================================================
## C.b. cell density
## --- parameters from first group (co-culture & pure culture+AH2QDS)
X    <-    c(23730717914.0633,    20555085940.8377,    13644400832.7275,
17438675361.1658, 7317926771.21383)             # estimated means
X.sd   <-   c(12261607884.2846,   3739844825.70899,   9406366306.16818,
2477759209.68928, 1366226947.91001)               # estimated standard
deviations
mean.X <- mean(X)                      # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## --------------------------------------------------------
## --- parameters from second group (pure culture)
Y <- c(4144933003.40598)                     # estimated means
Y.sd  <-  c(2538961720.08603)                     #  estimated  standard
deviations
mean.Y <- mean(Y)                        # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))           # standard deviation of mean.Y
## --------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## --------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
```

```
## Ha: difference (Z) not = 0
p.value <- 2*(1-pnorm(abs(Z)/sd.Z))
p.value


## ======================================================
## G.m. cell density
## --- parameters from first group (co-culture & pure culture+AH2QDS)
X <- c(495479855, 361184847, 95400467)             # estimated means
X.sd <- c(232186342, 205348896, 107232844)          # estimated standard
deviations
mean.X <- mean(X)                      # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## ------------------------------------------------------
## ---- parameters from second group (pure culture)
Y <- c(20081756)                    # estimated means
Y.sd <- c(21455033)                     # estimated standard deviations
mean.Y <- mean(Y)                       # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))          # standard deviation of mean.Y
## ------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## ------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
## Ha: difference (Z) not = 0
```

```
p.value <- 2*(1-pnorm(abs(Z)/sd.Z))
p.value


## ========================================================
##Substrate utilization extent
## -- parameters from first group (co-culture)
X <- c(0.831, 0.784, 0.761)             # estimated means
X.sd <- c(0.05, 0.06, 0.02)              # estimated standard deviations
mean.X <- mean(X)                      # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## --------------------------------------------------------
## --- parameters from second group (pure culture+ AH2QDS)
Y <- c(0.64, 0.669)                    # estimated means
Y.sd <- c(0.05, 0.05)                    # estimated standard deviations
mean.Y <- mean(Y)                       # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))          # standard deviation of mean.Y
## --------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## --------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
## Ha: difference (Z) not = 0
p.value <- 2*(1-pnorm(abs(Z)/sd.Z))
p.value
```

```
## ------------------------------------------------------
## --- parameters from first group (pure culture)
X <- c(0.441)            # estimated means
X.sd <- c(0.12)              # estimated standard deviations
mean.X <- mean(X)                    # mean of all estimated means
sd.mean.X <- sqrt(sum(X.sd^2)/length(X)) # standard deviation of mean.X
## ------------------------------------------------------
--- parameters from second group (pure culture+ AH2QDS)
Y <- c(0.64, 0.669)                  # estimated means
Y.sd <- c(0.05, 0.05)                  # estimated standard deviations
mean.Y <- mean(Y)                     # mean of all estimated means
sd.mean.Y <- sqrt(sum(Y.sd^2))          # standard deviation of mean.Y
## ------------------------------------------------------
##---  difference of both means
Z <-  mean.X - mean.Y
sd.Z <- sqrt(sd.mean.X^2 + sd.mean.Y^2)
## ------------------------------------------------------
## compute p-value of Z.test
## H0: difference (Z) = 0
## Ha: difference (Z) not = 0
p.value <- 2*(1-pnorm(abs(Z)/sd.Z))
p.value
```